UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Virus de salmónidos:
estudios sobre vacunas orales y prevención de persistencia viríca

PRESENTADA POR

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Memoria presentada por Natalia Andrea Ballesteros Benavides para optar al grado de doctor por la Universidad Complutense de Madrid, Madrid. 2015.

El trabajo recogido en esta memoria ha sido realizado en el Laboratorio de Virus de peces en el grupo de Vacunas y Expresión Génica del departamento de Microbiología Molecular y Biología de las Infecciones del Centro de Investigaciones Biológicas (CIB) del CSIC. Y ha sido financiado por fondos públicos a través de los proyectos AGL2010-18454 del Plan Nacional de I+D (Ministerio de Economía y Competitividad), y proyecto intramural CSIC 201020E084. Además Natalia A. Ballesteros Benavides ha sido beneficiaria de una beca predoctoral FPI del Ministerio de Ciencia e innovación BES-2011-048441.

Sylvia Rodríguez Saint-Jean y Sara I. Pérez Prieto, Investigadoras Titulares Centro de Investigaciones Biológicas (CSIC).

INFORMAN:

Que la presente tesis doctoral titulada "Virus de salmónidos: Estudios sobre vacunas orales y prevención de persistencia vírica." Que presenta Dña. Natalia Andrea Ballesteros Benavides para optar al grado de Doctor en Ciencias Veterinarias, ha sido realizada en el laboratorio de Virus de peces del grupo de Vacunas y Expresión Génica, departamento de Microbiología Molecular y Biología de las Infecciones del Centro de Investigaciones Biológicas, CSIC, bajo nuestra dirección, reuniendo los requisitos exigidos y considerando que está concluida, autorizan su presentación para que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, firmamos el presente informe en Madrid, 14 de Abril de 2015.

Fdo.: Sylvia Rodríguez Saint-Jean   Fdo.: Sara I. Pérez Prieto

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A mi familia

Resumen:

La Necrosis pancreática infecciosa (IPN) es una enfermedad viral grave de salmónidos. Es causada por virus de la necrosis pancreática infecciosa (IPNV), que es un miembro de la familia Birnaviridae. Recientemente se ha descrito una vacuna DNA por vía oral para trucha arco iris (*Oncorhynchus mykiss*) que protege e induce anticuerpos neutralizantes frente a IPNV. Debido a que los perfiles de transcripción de la respuesta génica no se conocían, nosotros los estudiamos en tejidos del riñón anterior (un órgano interno de respuesta inmune) y en un tejido de contacto con la vacuna (ciego pilórico), mediante el uso de un microarray de oligos enriquecido en genes relacionados con inmunidad y validados por RTqPCR. La cantidad de transcritos sobre-expresados fue mayor en tejidos de riñón anterior que en ciego pilórico, mientras que el número de transcritos sub-expresados o inhibidos fue mayor en el ciego pilórico que en el riñón anterior.

Seguidamente, se estudiaron en varios órganos los perfiles de respuesta transcripcional en truchas a diferentes tiempos post vacunación. Los perfiles también se compararon con los obtenidos después de la infección con IPNV. Un grupo de genes relacionados con la inmunidad (STAT1, IFN-I, IFNγ, Mx1, Mx3, IL8, IL10, IL11, IL12B, TNF2, MHC-I, IgM e IgT) se seleccionaron y analizaron por RTqPCR. Los resultados mostraron que la vacunación de pcDNA-VP2 por vía oral imitaba cualitativamente los perfiles de transcripción obtenidos tras la infección con IPNV, tanto a través del tiempo como en los órganos (riñón anterior, bazo, intestino, ciego pilórico, y el timo). Sin embargo, los niveles de expresión diferencial en la transcripción de la mayoría de los genes mencionados anteriormente fueron menores en las truchas vacunadas con pcDNA-VP2 que en las truchas infectadas con IPNV, a excepción de IFN-I que fue similar. En conjunto todos estos resultados sugieren que el proceso de inmunización de las truchas con la vacuna oral frente a IPNV ocurre de forma similar al proceso de infección con IPNV, aunque todavía se pueden lograr mejoras adicionales en el procedimiento de vacunación oral.

De acuerdo con los perfiles de respuesta transcriptómica del intestino de trucha existen muchos problemas sin resolver. Aún no se sabe cómo se regula la inmunidad intestinal en los teleósteos. Nosotros hemos estudiado la regulación de varios genes de respuesta inmune a lo largo de cinco segmentos del tracto digestivo, comparando los efectos observados en respuesta a una infección de IPNV con los provocados por la vacunación con pcDNA-VP2 por vía oral. Nos hemos centrado en la regulación de varias quimioquinas descritas en mucosas, receptores de quimioquinas, complejo mayor de histocompatibilidad II (MHC-II) y el factor de necrosis tumoral α

(TNF-α). Y además en el reclutamiento de células IgM(+) y CD3(+) a lo largo de los diferentes segmentos en respuesta a la infección con IPNV, utilizando técnicas de inmunohistoquímica. Nuestros resultados proporcionan evidencias de que existe una regulación diferencial de estos genes inmunitarios en respuesta a los estímulos a lo largo de los segmentos del intestino. Junto con esta inducción de quimioquinas y receptores de quimioquinas, el IPNV provocó una movilización de células IgM(+) e IgT(+) a las regiones del intestino anterior y ciego pilórico, y de células CD3(+) a las regiones del ciego pilórico e intestino medio y posterior.

Por lo tanto, nos centramos en la localización de las células B IgM(+) e IgT(+) en el tracto digestivo y su papel durante el curso de un estímulo local. Así, estudiamos la respuesta de las células B en estos cinco segmentos diferentes del tracto digestivo, tanto en los peces sin ningún tratamiento como en peces vacunados con pcDNA-VP2 por vía oral. Las células IgM(+) e IgT(+) fueron identificadas en el tracto intestinal con excepción del estómago en los peces sin tratamiento. Mientras que las células IgM(+) se localizaron en la lámina propia (LP), las células IgT(+) se localizaron principalmente como linfocitos intraepiteliales (IEL). Los IgM(+) IELs solo fueron detectados en el ciego pilórico. En respuesta a la vacunación oral, la región del ciego pilórico fue el área del tracto digestivo donde se demostró por RT-qPCR un mayor reclutamiento de células B, y por inmunohistoquímica se observó un aumento significativo de células IgM(+) e IgT(+) IELs. Nuestros resultados demuestran que tanto IgM(+) como IgT(+) respondieron ante la estimulación oral contradiciendo el paradigma de que los IELs de teleósteos son exclusivamente de células T. Inesperadamente, también hemos detectado células B en el tejido adiposo asociado al tracto digestivo que responde a la vacunación, sugiriendo que estas células rodeadas por los adipocitos también juegan un papel en la inmunidad de mucosa. Nuestros resultados contribuirán a una mejor comprensión de cómo la inmunidad de la mucosa está orquestada en los diferentes segmentos del intestino de los teleósteos.

El siguiente paso de nuestro trabajo, fue incorporar la vacuna pcDNA-VP2 en el pienso de la trucha y evaluar la eficacia de este método de administración oral de la vacuna. Para ello, se añadieron los complejos de plásmido recubierto con alginato liofilizado al pienso disuelto en agua, y tras ello la mezcla fue liofilizada nuevamente. Las truchas que fueron alimentadas durante tres días consecutivos con pienso con vacuna se compararon con las que recibieron el plásmido vacío en el pienso o con truchas alimentadas con pienso comercial. La expresión del gen VP2 se detectó en tejidos de diferentes órganos de truchas alimentadas con pienso recubierto con pcDNA-VP2 (riñón, bazo, intestino y branquias) durante los 15 días del transcurso de tiempo experimental. Esta vacuna pcDNA-VP2 claramente induce una respuesta inmune innata y específica, estimulando significativamente la expresión de los genes IFN-I, IFN-γ, Mx1, IL8, IL12, IgM e IgT. Se observó protección, con tasas de supervivencia relativa de 78% al 85,9% en las truchas vacunadas, así como niveles detectables de anticuerpos neutralizantes anti-IPNV durante al menos 90 días. De hecho, la replicación de IPNV en los peces vacunados fue significativamente inhibida a los 45 días pi.

Por otra parte, con objeto de comprobar si era posible optimizar la eficiencia de la vacuna pcDNA-VP2 encapsulada en microesferas de alginato, probamos el complejo quitosano-alginato como otro método de recubrimiento del plásmido de expresión. Detectamos el transcrito VP2 por RTqPCR en varios tejidos de trucha vacunadas con ambos tipos de microesferas y la inducción de genes de respuesta inmune innata (IFN-I, Mx1, IL8, IL12). También, se evaluó la protección frente a IPNV, examinando los efectos protectores de ese tipo de microesferas (que contienen pcDNA-VP2) tras la infección con el virus. Nuestros resultados muestran que tras la utilización de ambos tipos de microesferas se detectó en varios órganos el transcrito del gen VP2, y las expresiones de genes inmunitarios en el riñón fueron similares. Por otra parte, no se observaron diferencias significativas en los porcentajes de mortalidades acumulativas entre ambos tipos de recubrimiento. Por lo tanto, las microesferas de alginato inducen una protección mayor que la obtenida cuando los peces fueron vacunados por microesferas de quitosano-alginato con 10μg de la vacuna de DNA (95% y 73.4% RPS, respectivamente).

También se utilizó la vacuna pcDNA-VP2 por vía oral para entender mejor el estado de portador de IPNV y la acción de la vacuna. La eficacia de la vacuna se evaluó mediante la prevención de la persistencia del virus en los peces vacunados que sobrevivieron después de la exposición al virus por inmersión. Un análisis mediante RTqPCR reveló niveles más bajos en la transcripción del gen VP4 del IPNV en los peces vacunados y supervivientes a la infección respecto al grupo de peces supervivientes a la infección a los 45 dpi. Se recuperó virus infectivo en peces supervivientes asintomáticos (peces control virus), pero no de los peces vacunados supervivientes, lo que sugiere un papel activo de la vacuna en el control de la infección por IPNV. Por otra parte, los niveles de IPNV y la expresión de genes de la respuesta inmune se cuantificaron en los peces que presentaron una infección clínica, así como en los peces supervivientes asintomáticos a la infección. La vacuna imito la acción del virus, aunque, excepto en IFN-I e IL12, se detectó mayor expresión de los genes relacionados con la inmunidad en los peces supervivientes del grupo control virus (portadores) respecto al grupo de peces vacunados e infectados. Los niveles de transcripción de los genes examinados también mostraron diferencias significativas en los peces a los 10 y 45 días después de la infección.

El virus de la necrosis hematopoyética infecciosa (IHNV) es el agente etiológico de una enfermedad viral grave que causa la infección sistémica y alta mortalidad en salmónidos. Por inyección intramuscular, una vacuna DNA "todo pez" (pIRF1A-G) que tiene como promotor el factor regulador de interferón 1A (IRF1A) de trucha arco iris y que expresa la glicoproteína G del IHNV, induce respuesta inmune protectora a trucha. Sin embargo, es necesario encontrar otras rutas de administración de vacunas que sean más fáciles, más ajustada la relación coste/efectividad y que permitan administrarse a gran número de alevines a las edades que son susceptibles. En este estudio, la vacuna pIRF1A- fue encapsulada en microesferas de alginato y administrada en trucha por vía oral. A los 1, 3, 5, y 7 dpv. se detectó por RTqPCR la transcripción del gen G del IHNV en las branquias, bazo, riñón e intestino de truchas vacunadas por vía oral con 10 µg de pIRF1A-G. Estos resultados sugieren que la encapsulación de pIRF1A-G en alginato asegura que la vacuna llegue al segundo segmento del intestino posterior, donde los antígenos son absorbidos. Además, pIRF1A-G indujo la expresión de varios genes marcadores de la respuesta inmune innata y adaptativa (IFN-I, Mx1, Mx3, Vig1, Vig2, TLR3, TLR7, TLR8, CD4, CD8, IgM e IgT) en tejido de riñón y bazo de truchas inmunizadas oralmente con 10, 25 y 100 µg de pIRF1A-G. Nuestros resultados demostraron que la administración de pIRF1A-G por vía oral requiere aproximadamente 20 veces más DNA plasmídico que la vacunación por inyección intramuscular para inducir una respuesta inmune protectora similar. El efecto dosis-respuesta se hizo evidente cuando los peces vacunados con 10, 20, 25 y 50 µg de pcIRF1A-G se infectaron por inmersión con IHNV, obteniéndose valores de supervivencia relativa (RPS) de 21, 30, 30 y 45% respectivamente. La administración oral de una dosis de 100 µg indujo una protección similar a la obtenida cuando los peces se vacunaron con 5 µg por vía im. (RPS 56 y 67 respectivamente). También detectamos un aumento de anticuerpos anti-IHNV en suero de peces vacunados oralmente con dosis altas (50 o 100 µg) de pIRF1A-G.

Resumiendo, nuestros resultados demostraron un aumento significativo y dosis-dependiente de respuesta inmune y resistencia a la infección con virus IHN, en peces vacunados con pIRF1A-G encapsulada en microesferas de alginato y administrada por vía oral.

En el caso de la modulación de la infección IHNV, la información sobre la respuesta de la célula huésped al virus es aún limitada. En nuestro trabajo hemos caracterizado la respuesta transcripcional de la trucha arco iris infectada con IHNV, mediante la realización de análisis utilizando secuenciación masiva Illumina (RNA-seq).

El ensayo de RNA-seq fue realizado en el tejido del riñón anterior, en dos tiempos diferentes tras la infección, que representan diferentes etapas de la enfermedad. La referencia utilizada fueron los genes ohnologos y genes no-ohnologos, y una vez realizado el análisis de los datos, las isoformas de los genes ohnologos y no-ohnologos fueron combinadas y se informaron como transcritos totales. Un total de 6875 transcritos estadísticamente diferenciados (DETs) y 5857 DETs fueron identificados en respuesta al IHNV a los 3 y 7 días post-infección (dpi) respectivamente, en comparación con los peces control no infectados; 3141 de los DETs fueron comunes en ambos, 3 y 7 dpi. De ellos, se encontró un total de 340 DETs con un nivel de expresión (fold change)> 2 o <0,5 en relación al control de truchas no infectadas. Al menos un término GO anotado pudo ser asignado a la mayoría de los DETs (173 de 340).

Estos DETs se clasificaron en transcripciones que participaron en ambos eventos tanto a los 3 y 7 dpi (47 DETs), y transcripciones que se modularon únicamente en un sólo punto de tiempo después de la infección (198 DETs regulados a los 3 dpi y 18 DETs a los 7 dpi). Además, la anotación funcional por KEGG Orthology mostró 58 vías metabólicas (“pathways”) que contienen genes modulados tanto a los 3 como a los 7 dpi; 170 vías metabólicas activadas de forma única a los 3 dpi y 34 vías metabólicas a los 7 dpi. De estos, examinamos las vías metabólicas o “pathways” TLR, JAK-STAT y Apoptosis y representamos gráficamente los genes sobre-regulados y sub-regulados.

Además otras vías inmunológicas interesantes, tales como la vía de señalización de quimioquinas, diferenciación de células hematopoyéticas, interacción de citoquinas y receptores-citoquinas, vía de señalización de TNF, y células asesinas (Natural Killer) se ilustraron para ser mostradas como material complementario.

Abstract:

Infectious pancreatic necrosis (IPN) is a severe viral disease of salmonid fish. It is caused by infectious pancreatic necrosis virus, which is a member of the Birnaviridae family. Induction of neutralizing antibodies and protection by oral vaccination with DNA-alginates of rainbow trout (*Oncorhynchus mykiss*) against IPNV was recently reported. Because orally induced immune response transcript gene profiles had not been described yet neither in fish, nor after IPNV vaccination, we studied them in head kidney (an immune response internal organ) and a vaccine entry tissue (pyloric caeca). By using an oligo microarray enriched in immune-related genes validated by RTqPCR, the number of increased transcripts in head kidney was higher than in pyloric caeca while the number of decreased transcripts was higher in pyloric caeca than in head kidney.

Later, time-course and organ transcriptional response profiles in trout were studied after oral pcDNA-VP2 vaccination. The profiles were also compared with those obtained after infection with IPNV. A group of immune-related genes (STAT1, IFN-I, IFNγ, Mx1, Mx3, IL8, IL10, IL11, IL12b, TNF2, MHC-I, IgM and IgT) previously selected from microarray analysis of successful oral vaccination of trout, were used for the RTqPCR analysis. The results showed that oral VP2-vaccination qualitatively mimicked both the time-course and organ (head kidney, spleen, intestine, pyloric caeca, and thymus) transcriptional profiles obtained after IPNV-infection. However, transcriptional differential expression levels of most of the genes mentioned above were lower in VP2-vaccinated than in IPNV-infected trout, except for IFN-I which were similar. Together all the results suggest that the oral-alginate VP2-vaccination procedure immunizes trout against IPNV in a similar way as IPNV-infection does while there is still room for additional improvements in the oral vaccination procedure.

According with the gut transcriptome response profiles in trout there are still many details of how intestinal immunity is regulated that remain unsolved in teleost. We have studied the regulation of several immune genes along five segments of digestive tract, comparing the effects observed in response to an IPNV infection to those elicited by oral pcDNA-VP2 vaccination. We have focused on the regulation of several mucosal chemokines, chemokine receptors, the major histocompatibility complex II (MHC-II) and tumor necrosis factor α (TNF-α). Furthermore, the recruitment of IgM(+) cells and CD3(+) cells was evaluated along the different segments in response to IPNV by immunohistochemical techniques. Our results provide evidences that there is a differential regulation of these immune genes in response to

both stimuli along the gut segments. Along with this chemokine and chemokine receptor induction, IPNV provoked a mobilization of IgM(+) and IgT(+) cells to the foregut and pyloric caeca region, and CD3(+) cells to the pyloric caeca and midgut/hindgut regions.

Therefore, we focused on the location of IgM(+) and IgT(+) B cells along the digestive tract and their role during the course of a local stimulus. Thus, we have studied the B cell response in those five different segments of the digestive tract in both naïve trout and fish orally vaccinated pcDNA-VP2. IgM(+) and IgT(+) cells were identified all along the tract with the exception of the stomach in naïve fish. While IgM(+) cells were mostly located in the lamina propria (LP), IgT(+) cells were primarily localized as intraepithelial lymphocytes (IELs). Scattered IgM(+) IELs were only detected in the pyloric caeca. In response to oral vaccination, the pyloric caeca region was the area of the digestive tract in which a major recruitment of B cells was demonstrated through both real time PCR and immunohistochemistry, observing a significant increase in the number of both IgM(+) and IgT(+) IELs. Our findings demonstrate that both IgM(+) and IgT(+) respond to oral stimulation and challenge the paradigm that teleost IELs are exclusively T cells. Unexpectedly, we have also detected B cells in the fat tissue associated to the digestive tract that respond to vaccination, suggesting that these cells surrounded by adipocytes also play a role in mucosal defense. Our results will contribute to a better understanding of how mucosal immunity is orchestrated in the different gut segments of teleost.

The next step of our work was incorporated into feed to evaluate the effectiveness of this oral delivery method in trout. Lyophilized alginate-plasmid complexes were added to feed dissolved in water and the mixture was then lyophilized again. We compared trout that were fed for 3 consecutive days with vaccine pellets with fish that received the empty plasmid or a commercial pellet. VP2 gene expression could be detected in tissues of different organs in the trout that received the pcDNA-VP2 coated feed (kidney, spleen, gut and gill) throughout the 15 day time-course of the experiments. This pcDNA-VP2 vaccine clearly induced an innate and specific immune-response, significantly up-regulating IFN-I, IFN-γ, Mx1, IL8, IL12, IgM and IgT expression. Strong protection, with relative survival rates of 78%-85.9% were recorded in the vaccinated trout, which produced detectable levels of anti-IPNV neutralizing antibodies during 90 days at least. Indeed, IPNV replication was significantly down-regulated in the vaccinated fish 45 days pi.

Moreover, we tested chitosan-alginate complex to prove if this encapsulation would optimize the DNA vaccine delivery better than single alginate does. The VP2 gene expression was detected in several organs of trout by using both types of microspheres, as well as the induction of some genes considered markers of expression of immune innate response (IFN-I, Mx1, IL8, IL12). The protection against IPNV was also evaluated by examining the protective effects of both, alginate and alginate/chitosan microspheres than contain pcDNA-VP2. Our results show that using any of the microspheres, the VP2 transcript gene expression was detected in several organs and the expression levels of immune genes in kidney were similar. On the other hand not significant differences were observed in cumulative percent mortalities (CPM) in fish vaccinated with those two encapsulation types. Thus, alginate microspheres induced better protection than that obtained when fish were vaccinated with 10µg of the DNA vaccine chitosan-alginate microspheres (95% and 73.4% RPS, respectively).

The oral pcDNA-VP2 vaccine was also used for oral DNA vaccination of fish to better understand the carrier state and the action of the vaccine. The efficacy of the vaccine was evaluated by measuring the prevention of virus persistence in the vaccinated fish that survived after waterborne virus challenge. A real-time RTqPCR analysis revealed lower levels of IPNV-VP4 transcripts in trout survivors among vaccinated and challenged fish compared with the control virus group at 45 dpi. The infective virus was recovered from asymptomatic virus control fish, but not from the vaccinated survivor fish, suggesting an active role of the vaccine in the control of IPNV infection. Moreover, the levels of IPNV and immune-related gene expression were quantified in fish showing clinical infection as well as in asymptomatic rainbow trout survivors. The vaccine mimicked the action of the virus, although stronger expression of immune-related genes, except for IFN-I and IL12, was detected in survivors from the virus control (carrier) group than in those from the vaccinated group. The transcriptional levels of the examined genes also showed significant differences in the virus control fish at 10 and 45 days post-challenge.

The rhabdovirus Infectious haematopoietic necrosis virus (IHNV) is the etiological agent of a severe viral disease that causes systemic infection and significant mortality in a variety of salmonid fish. In case of intramuscular injection, an all-fish DNA vaccine (pIRF1A-G) containing the rainbow trout interferon regulatory factor 1A (IRF1A) promoter driven the expression of the infectious hematopoitic necrosis virus (IHNV) glycoprotein (G) elicited protective immune responses in trout. However, cost effective, less laborious, alternative routes of DNA vaccine delivery are required to vaccinate large numbers of susceptible small fish. In this study, the pIRF1A-G vaccine was encapsulated into alginate microspheres and orally administered to rainbow trout. At 1, 3, 5, and 7 dpv, IHNV G transcripts were detected by RTqPCR in the gills, spleen, kidney and intestinal tissues of fish orally vaccinated with 10 µg of pIRF1A-G. These results suggested that the encapsulation of pIRF1A-

G in alginate microparticles ensured that the DNA vaccine reached the second segment of the hind gut where antigens are absorbed. Moreover, pIRF1A-G induced the expression of several markers of the innate and adaptive immune responses (IFN-I, Mx1, Mx3, Vig1, Vig2, TLR3, TLR7, TLR8, CD4, CD8, IgM and IgT) in kidney and spleen tissues of fish orally immunized with 10, 25 and 100 µg of pIRF1A-G. Interestingly, our results demonstrated that the oral administration of pIRF1A-G requires approximately 20-fold more plasmid DNA than the vaccination by intramuscular injection to induce similar protective immune responses. When vaccinated fish were challenged by immersion with live IHNV, a dose-response effect was evident for the fish orally vaccinated with 10, 20, 25 and 50 µg of pIRF1A-G, which exhibited relative percent survival (RPS) values of 21, 30, 30 and 45, respectively. Although the protective effects of pIRF1A-G after challenge were relatively modest, significant differences CPM among the fish orally vaccinated with 10, 20, 25 and 50 µg and the unvaccinated or empty-plasmid vaccinated fish were observed. Oral administration of a higher dose of pIRF1A-G (100 µg) induced significant protection more similar to that obtained when fish were vaccinated by intramuscular injection with 5 µg of the DNA vaccine(56 and 67 % RPS, respectively). We also detected an increase in anti-IHNV antibodies in serum samples of fish orally vaccinated with alginate microspheres containing high doses of pIRF1A-G (50 or 100 µg) when compared with unvaccinated fish. In summary, our results demonstrated a significant increase induced of dose-dependent immune responses and resistance to an IHNV infection after the oral administration of DNA vaccine encapsulated in alginate microspheres.

On the other hand, ccurrently, there is only limited information on the host cell response to the IHNV and we were interested in this approach. Our work characterized the transcriptional response of rainbow trout to IHNV infection by conducting a high throughput RNA-seq analysis using Illumina next-generation sequencing. RNA-seq was performed on the anterior kidney tissue of rainbow trout at two time points post-infection that represent different stages of the disease. Duplicate gene isoforms from orthologous references and unique genes from non-ohnologous isoforms were then combined and reported as the total transcripts. Once the isoforms were combined, a total of 6875 differentially expressed transcripts (DETs) and 5857 DETs were identified in response to IHNV at 3 and 7 days post-infection (dpi), respectively, compared with uninfected control fish; 3141 of the DETs were common to the fish analyzed at each time point. A total of 340 DETs were found in 3 dpi and 7 dpi samples with a fold change > 2 or < 0.5 relative to control fish. At least one annotated GO term could be assigned to most of the DETs (173 out of 340). The filtered DETs were classified into transcripts involved in events at both 3 and 7 dpi (47 DETs) and transcripts that were modulated at just one of the post-infection time points (198 DETs up-regulated at 3 dpi and 18 at 7 dpi). In addition, functional annotation by KEGG Orthology rendered 58 pathways that contain modulated genes at both 3 and 7 dpi, 170 pathways activated uniquely at 3 dpi and 34 at 7 dpi. Of these, TLR, JAK-STAT and apoptosis pathways were further examined, and the highlighted up- or down-regulated genes were graphically represented. In addition, interesting immune pathways, such as the chemokine signaling pathway, hematopoietic cell differentiation, cytokine-cytokine receptor interaction, the TNF signaling pathway, and natural killer cell mediated cytotoxicity, are shown in the supplementary materials.

Publicaciones:

- Immune responses to oral pcDNA-VP2 vaccine in relation to infectious pancreatic necrosis virus carrier state in rainbow trout *Oncorhynchus mykiss*. Ballesteros NA, Saint-Jean SS, Perez-Prieto SI, *Veterinary Immunology and Immunopathology* Accepted Marzo 2015

- Gene2Path: A Data analysis tool to study fish gene pathways by automatic search of orthologous genes. Ballesteros NA, Aguirre N. F, Coll J, Perez-Prieto SI, Rodriguez Saint-Jean S, *J. Aquac Res Development* 2015 6:329 doi:10.4172/2155-9546.1000329

- Food pellets as an effective delivery method for a DNA vaccine against infectious pancreatic necrosis virus in rainbow trout (*Oncorhynchus mykiss, Walbaum*). Ballesteros NA, Rodriguez Saint-Jean S, Perez-Prieto SI. *Fish Shellfish Immunol.* 2014 Apr;37(2):220-8. doi: 10.1016/j.fsi.2014.02.003. Epub 2014 Feb 21.

- Modulation of genes related to the recruitment of immune cells in the digestive tract of trout experimentally infected with infectious pancreatic necrosis virus (IPNV) or orally vaccinated.Ballesteros NA, Rodríguez Saint-Jean S, Pérez-Prieto SI, Aquilino C, Tafalla C. *Dev Comp Immunol.* 2014 May;44(1):195-205. doi: 10.1016/j.dci.2013.12.009. Epub 2013 Dec 24.

- The pyloric caeca area is a major site for IgM(+) and IgT(+) B cell recruitment in response to oral vaccination in rainbow trout. Ballesteros NA, Castro R, Abos B, Rodríguez Saint-Jean SS, Pérez-Prieto SI, Tafalla C. *PLoS One.* 2013 Jun 13;8(6):e66118. doi: 10.1371/journal.pone.0066118. Print 2013.

- Trout oral VP2 DNA vaccination mimics transcriptional responses occurring after infection with infectious pancreatic necrosis virus (IPNV). Ballesteros NA, Saint-Jean SS, Perez-Prieto SI, Coll JM. *Fish Shellfish Immunol.* 2012 Dec;33(6):1249-57. doi: 10.1016/j.fsi.2012.09.004. Epub 2012 Oct 3.

- Oral immunization of rainbow trout to infectious pancreatic necrosis virus (IPNV) induces different immune gene expression profiles in head kidney and pyloric ceca. Ballesteros NA, Saint-Jean SS, Encinas PA, Perez-Prieto SI, Coll JM. *Fish Shellfish Immunol.* 2012 Aug;33(2):174-85. doi: 10.1016/j.fsi.2012.03.016. Epub 2012 Apr 10.

Publicaciones enviadas:

- An oral DNA vaccine against infectious haematopoietic necrosis virus (IHNV) encapsulated in alginate microspheres induces dose-dependent immune responses and significant protection in rainbow trout (*Oncorrhynchus mykiss*). Natalia A. Ballesteros, Marta Alonso, Sylvia Rodríguez Saint-Jean and Sara I Perez-Prieto, manuscrito para ser enviado a *Fish Shellfish Immunol.* 2015

- RNA-seq profiles from rainbow trout kidney tissue after infectious haematopoietic necrosis virus (IHNV) infection at early (asymptomatic) and symptomatic time points. Natalia A. Ballesteros, Haydee Artaza, Guillermo Padilla, Dolores Alonso, Sylvia Rodríguez Saint-Jean and Sara I Perez-Prieto, manuscrito para ser enviado a *Veterinary Research* 2015.

Índice

Abreviaturas.

ºC	grados centígrados
DNA	ácido desoxirribonucleico
cDNA	ácido desoxirribonucleico complementario
RNA	ácido ribonucleico
mRNA	ácido ribonucleico mensajero
CC	control células
CI_{50}	concentración inhibitoria 50%
CpGs	motivos no metilados de citosinas y guaninas
CT_{50}	concentración tóxica 50
CV	control virus
dNTP	desoxirribonucleótidos trifosfato
DO	densidad óptica
DTT	diclorodifeniltricloroetano
ECP	efectos citopáticos
EDTA	etilen diamino tetra-Acético
ELISA	ensayo por inmunoabsorción ligado a enzimas / "*Enzyme-Linked ImmunoSorbent Assay*"
et al.	*et álii* (locución latina "y otros")
FITC	isotiocianato de fluoresceína
h	horas
IFN	interferón
IHN	necrosis hematopoyética infecciosa
IHNV	virus de la necrosis hematopoyética infecciosa
IPN	necrosis pancreática infecciosa
IPNV	virus de la necrosis hematopoyética infecciosa
l	litros
MOI	multiplicidad de infección

MHC	complejo mayor de histocompatibilidad
min	minutos
OIE	Organización Mundial de Sanidad Animal
ORF	marco de lectura abierta
pb	pares de bases
PBS	tampón fosfato salino
CMV	Promotor de un plasmido, citomegalovirus
IRF	Promotor de un plasmido, IRF
pcDNA	plásmido de expresión de DNA no codificante utilizado como control negativo, posee un promotor CMV.
pcDNA-VP2	plásmido de expresión de DNA con el inserto de la proteína VP2 utilizado como vacuna de DNA frente a IPNV, posee un promotor CMV.
pcIRF	plásmido de expresión de DNA no codificante utilizado como control negativo, posee un promotor IRF.
pcIRF-G	plásmido de expresión de DNA con el inserto de la proteína G utilizado como vacuna de DNA frente a IHNV, posee un promotor IRF.
PCR	reacción en cadena de la polimerasa
qPCR	reacción en cadena de la polimerasa en tiempo real
PEG	polietilenglicol
pi	post-infección
PM	peso molecular
ppm	partes por millón
pv	post-vacunación
rpm	revoluciones por minuto
PRS	porcentaje relativo de supervivencia
RTqPCR	transcripción inversa acoplada a la reacción en cadena de la polimerasa
SDS	dodecil sulfato de sodio

seg.	segundos
SF	suero fetal bovino
t.a.	temperatura ambiente
TAE	tampón Tris/ acetato/ EDTA
TBS	tampón Tris salino
$TCID_{50}$	dosis infectiva 50% en cultivos celulares
Tris	tris (hidroximetil)- aminometano
UI	unidades internacionales
VHS	septicemia hemorrágica viral
VHSV	virus de la septicemia hemorrágica viral
FPKM	Fragments per kilobase of exon per millon fragments mapped
MHC	Complejo mayor de histocompatibilidad
ICOS	Coestimulador inducible ICOS
TH	Subconjunto de células T helper (TH1, TH2 y TH17)
NCBI	Base de datos del National Center for Biotechnology Information
KEGG	Base de datos de Kyoto Encyclopedia of Genes and Genomes
DETs	Transcritos estadísticamente diferenciados
IDs	Numero o código Identificador.

Introducción

Virus y Acuicultura

Los virus son entidades subcelulares que parasitan la maquinaria biosintética y genética celular (DNA, RNA y proteínas) que es utilizada para su replicación intracelular. Como resultado se producen los viriones, que constituyen la fase extracelular de los virus (Carrasco and Almendral 2006). Los primeros experimentos con virus (a finales del siglo XIX y comienzos del XX) se diseñaron para separarlos de los microbios que podían ser observados en el microscopio óptico y que generalmente se cultivaban en medios sencillos. Durante años la única característica que identificaba como virus a un microorganismo era, dado su pequeño tamaño, su condición de filtrarse a través de filtros que sí retenían bacterias; la mayoría de los estudios sobre virus se centraban en su capacidad para producir infecciones y enfermedades. Por eso los primeros esfuerzos para clasificar virus se basaron en las propiedades patogénicas observadas, así como en tropismos hacia órganos determinados y en las características ecológicas y de transmisión. Y estas características eran reflejadas en la denominación de estos microorganismos. La acuicultura es uno de los sectores de producción de alimento de más rápido crecimiento a nivel mundial. En la década de 1970, suponía alrededor del 6% del pescado disponible para el consumo; en 2012 esta cifra fue del 50%. Se prevé que en el año 2030 la acuicultura proporcione alrededor de un 62% del pescado destinado a la alimentación humana mundial (FAO 2014).

Las enfermedades infecciosas se consideran cada vez más como una importante restricción para la producción en acuicultura y están afectando al desarrollo económico del sector en numerosos países. Los recursos tradicionales frente a una infección por microorganismos en acuicultura son la prevención y el control de la dispersión del patógeno, la aplicación de técnicas diagnósticas rápidas y fiables para la detección precoz que permita contener el brote y la aplicación de vacunas eficaces. En lo que respecta al cultivo de peces, la obtención de unos niveles satisfactorios de salud así como niveles de contaminación bajos, son prioritarias para el mantenimiento de una instalación. Así lo demuestra la preocupación de la OIE y Unión Europea, donde las normativas para la sanidad animal y prevención de enfermedades en acuicultura, se han reforzado y unificado mediante revisión frecuente (OIE 2012). En acuicultura piscícola cabe destacar el cultivo de Salmónidos, que ha sido el gran protagonista en la acuicultura europea y Americana. Chile junto con Noruega son los primeros productores mundiales de salmón.

En las décadas 1950-1960 se produjo la mayor “explosión” en el descubrimiento de nuevos virus. En el caso de los peces Teleósteos, en 1941 se describió por primera vez una enfermedad que afectaba a alevines de trucha, causando una enteritis catarral aguda que fue atribuida a problemas nutricionales. Años más tarde, un estudio histopatológico demostró evidentes lesiones en páncreas, y la descripción de una enfermedad de origen infeccioso que se denominó enfermedad de la necrosis pancreática infecciosa (IPN) postulada como posible origen viral (Wood, Snieszko, and Yasutake 1955). En este intervalo de tiempo se habían desarrollado por primera vez métodos para el cultivo de células de peces y en los años 50 se aisló de trucha arco iris (*Oncorhynchus mykiss)* el primer virus de un pez teleósteo que se cultivó en cultivos celulares, el virus de la necrosis pancreática infecciosa (en adelante IPNV). Este aislamiento fue presentado en un Congreso y seguidamente publicado (Wolf et al. 1960). Wolf & Quimby (1962) (Wolf and Quimby 1962) establecieron la línea celular RTG-2 a partir de gónada de trucha arco iris y comprobaron su susceptibilidad al IPNV. Además del IPNV, los virus que producen mayores pérdidas económicas internacionalmente en salmónidos son el virus de la necrosis hematopoyética infecciosa (IHNV) y el virus de la septicemia hemorrágica viral (VHSV) que pertenecen a la familia Rhabdoviridae, género Novirhabdovirus (King et al. 2011). El virus IPN es de especial interés en España porque es el virus de mayor prevalencia en acuicultura (Rodriguez Saint-Jean, Vilas Minondo, and Perez Prieto 1994) y junto con el IHNV de gran importancia económica en USA y virus emergente en Europa. Son los virus que estudiaremos en ésta tesis.

El interés de los virus que afectan a peces teleósteos no ha decrecido en los últimos años ya que la acuicultura se ha expandido, intensificado y diversificado en todo el mundo, y los movimientos de animales vivos o sus productos, han acelerado la dispersión accidental de enfermedades en nuevas poblaciones y regiones geográficas. Entre las enfermedades que afectan a peces cultivados, aquellas cuyo agente etiológico es un virus, han resultado especialmente interesantes por las dificultades en el conocimiento de las características del patógeno, su ciclo biológico, reservorios y dificultades en el hallazgo de métodos de prevención y control (Wallace et al. 2008). En este contexto, los virus de Salmónidos, pese a ser los más estudiados en acuicultura, continúan siendo objeto de numerosas investigaciones porque existen demasiados interrogantes sobre su ciclo biológico, patogenia, y respuesta inmune. Todo ello importante para poder controlar la dispersión del patógeno, obtener estirpes de animales resistentes a la infección o conseguir vacunas eficaces, económicas y fácilmente aplicables.

Necrosis Pancreática Infecciosa (IPN).

La Necrosis Pancreática Infecciosa (IPN) es una enfermedad viral aguda y sistémica que afecta a salmónidos tanto en agua dulce como en agua de mar. En el caso de la trucha los alevines son más susceptibles poco después de la eclosión, alcanzando mortalidades desde el 10 hasta el 90% en las primeras 20 semanas; en salmones, también hay mortalidades en juveniles después de la transferencia al mar. Además de la mortalidad causada directamente por la infección viral, el virus también causa inmunosupresión, haciéndolos más vulnerables a otros patógenos (Reno 1999; Specter et al. 2009).

Sintomatología

Los síntomas de la enfermedad en salmónidos presentan características de enteritis catarral aguda, aunque no se han descrito signos patognomónicos de la enfermedad. Los peces presentan cambios en el comportamiento que incluyen anorexia y natación aberrante en círculos intercalados con ataxia. Respecto a los signos macroscópicos, es común encontrar melanosis, distensión abdominal, ascitis, exoftalmia leve a moderada, palidez en las branquias, y hemorragias en aletas y zona ventral (Rodriguez Saint-Jean, Borrego, and Perez-Prieto 2003). En la necropsia, el estómago e intestino no contiene alimento y en su lugar se observa un moco blanquecino; además en el tejido adiposo, ciego pilórico, intestino anterior y vísceras en general se pueden observar hemorragias petequiales. El bazo, corazón, hígado y riñones pueden presentar hipertrofia y palidez (Rodriguez Saint-Jean et al. 2003)

Histopatología

En el páncreas de peces infectados se observan lesiones compatibles con necrosis grave y masiva de células acinares con picnosis nuclear, cariorrexis e inclusiones basófilas citoplasmáticas (Smail et al. 1995; Taksdal, Stangeland, and Dannevig 1997). En el citoplasma de las células acinares pancreáticas, puede observarse partículas virales icosaédricas (Lightner and Post 1969). En el riñón anterior y posterior se observan zonas de congestión y hemorragias en los glomérulos, edema y destrucción del epitelio tubular (McKnight and Roberts 1976). La mucosa del tracto intestinal presenta signos de enteritis aguda con necrosis y desprendimiento del epitelio (Wood et al. 1955). En el hígado se puede observar congestión y necrosis con degeneración focal de las células del parénquima (Rodriguez Saint-Jean et al. 2003; Wood et al. 1955).

Patogénesis de la enfermedad

El tiempo de incubación de la Necrosis Pancreática Infecciosa depende de varios factores como son: la edad del huésped, la temperatura del agua y la especie afectada. En infecciones experimentales en trucha arco iris, se ha detectado el virión en células exocrinas pancreáticas a los dos días de infección, se desconoce si el virus entra en el páncreas directamente a través de la serosa o llega allí por vía sanguínea. El páncreas es el principal órgano afectado. También se han observado cambios patológicos en el riñón e hígado de peces infectados (Swanson and Gillespie 1981).

Los signos clínicos de la enfermedad comienzan a los 2 a 4 días post infección (Swanson and Gillespie 1982), la mayor mortalidad es entre 10-12 días y la duración de la enfermedad es 4-5 semanas. Los peces supervivientes a IPN pueden adquirir el estado de portadores asintomáticos, transmitiendo la enfermedad de forma horizontal y/o vertical (Rodriguez Saint-Jean, Perez-Prieto, and Vilas Minondo 1992). La transmisión horizontal de IPN es causada por peces clínicamente enfermos y/o peces portadores asintomáticos, quienes excretan virus a través de heces, orina, fluidos sexuales y la mucosidad externa (Rodriguez Saint-Jean et al. 2003). El virus se puede transmitir continuamente a otros peces (Bootland, Dobos, and Stevenson 1991; Rodriguez Saint-Jean et al. 2003) y esto puede constituir un mecanismo de supervivencia. Varios factores, incluyendo la edad, especie de peces, temperatura del agua, y la cepa viral, pueden influir en la gravedad de la infección y el posterior establecimiento del estado de portador (Mcallister et al. 1993; Sadasiv 1995). La persistencia del IPNV y el establecimiento del estado de portador puede ser debida a que el virus es capaz de infectar leucocitos y como consecuencia inhibir la respuesta inmune del hospedador (Swanson, Carlisle, and Gillespie 1982).

En el salmón atlántico (*Salmo salar*) se ha demostrado que el estado de portador asintomático puede mantenerse durante toda su vida (Melby et al. 1991), a pesar de que la replicación de IPNV es inhibida por la producción de interferón (IFN) y proteínas antivirales. Sin embargo, para mantener una condición de portador, el virus no puede ser completamente eliminado y debe existir un equilibrio entre la capacidad de replicación viral y los mecanismos de defensa del huésped. A nivel molecular, los cambios en los aminoácidos 217 y 221 (Pro-Thr) de la proteína de la cápside viral VP2, han sido involucrados en las características de virulencia y persistencia para el serotipo Sp. (Santi, Vakharia, and Evensen 2004; Song et al. 2005).

Virus de la Necrosis Pancreática Infecciosa (IPNV)

Morfología del virión

Este virus se clasificó como Reovirus en los primeros años de su aislamiento y descripción. Actualmente, se clasifica taxonómicamente en la familia Birnaviridae, género Aquabirnavirus (Van Regenmortel et al. 2000); es un virus con una cápsida icosaédrica sin envuelta de alrededor de 60 nm de diámetro (55-75 nm). La cápsida está formada por 180 subunidades estructurales compartidas entre 132 capsómeros en la superficie del virión que posee un número de triangulación (T) de 13 (Ozel and Gelderblom 1985). Ver Figura 1.

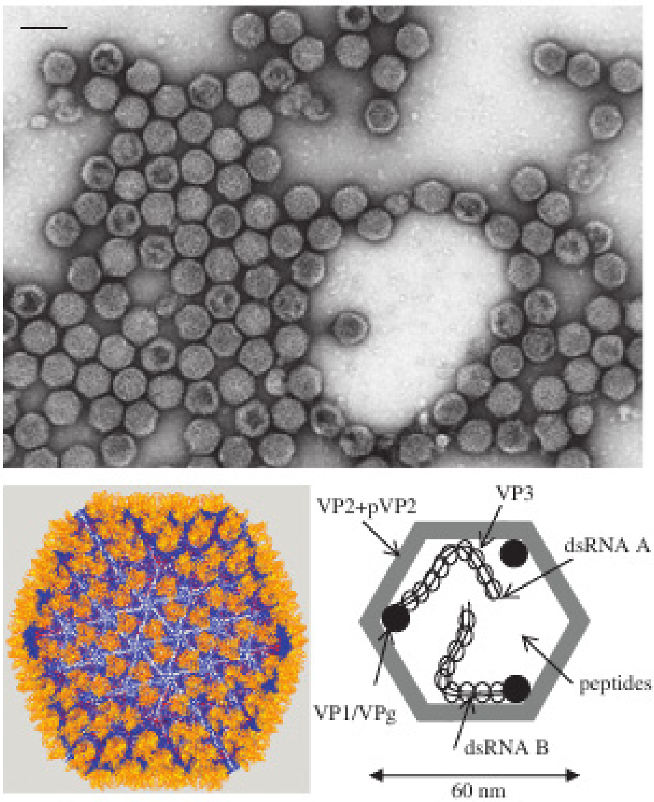

Figura 1: Parte Superior, estructura del virus IPNV, Microscopia electrónica del virus IPN (Cortesía de J. Ruiz Gastón, CNB, Madrid) barra en imagen superior corresponde a 100 nm. Y parte inferior, esquema de la partícula viral.

Genoma viral

El genoma es RNA de doble cadena y bisegmentado. El segmento A del genoma del IPNV tiene aproximadamente 3.100 pares de bases (pb) (Duncan and Dobos 1986) y presenta dos marcos de lectura abiertos (ORF). El ORF de mayor tamaño es de unas 2.900 pb, está flanqueado en los extremos 3' y 5' por regiones terminales de 119 y 62 bases respectivamente, las cuales se cree que puedan servir de unión para el ribosoma y el RNA polimerasa (Nagy et al. 1987). Este ORF codifica una poliproteína de 106 kDa (NH2-preVP2-VP4-VP3-COOH). El ORF de menor tamaño, de 17 kDa, se solapa con el mayor en el extremo amino terminal (Nagy et al. 1987), codificando un polipéptido no estructural rico en arginina, que fue detectado por primera vez en cultivos celulares infectados con el virus (Magyar and Dobos 1994).

El segmento B de aproximadamente 2.700 pb codifica una única proteína, la polimerasa viral RNA dependiente, conocida como VP1. Esta proteína constituye la RNA polimerasa del virión dependiente de RNA de doble cadena (RdRp) (Duncan et al. 1991) (Ver Figura 2). El contenido de GC del genoma viral es del 54% y la temperatura de desnaturalización es de 89ºC. El extremo 5' de cada hebra de RNA está ligado a un residuo de serina en la VPg por un enlace fosfodiéster (Calvert et al. 1991).

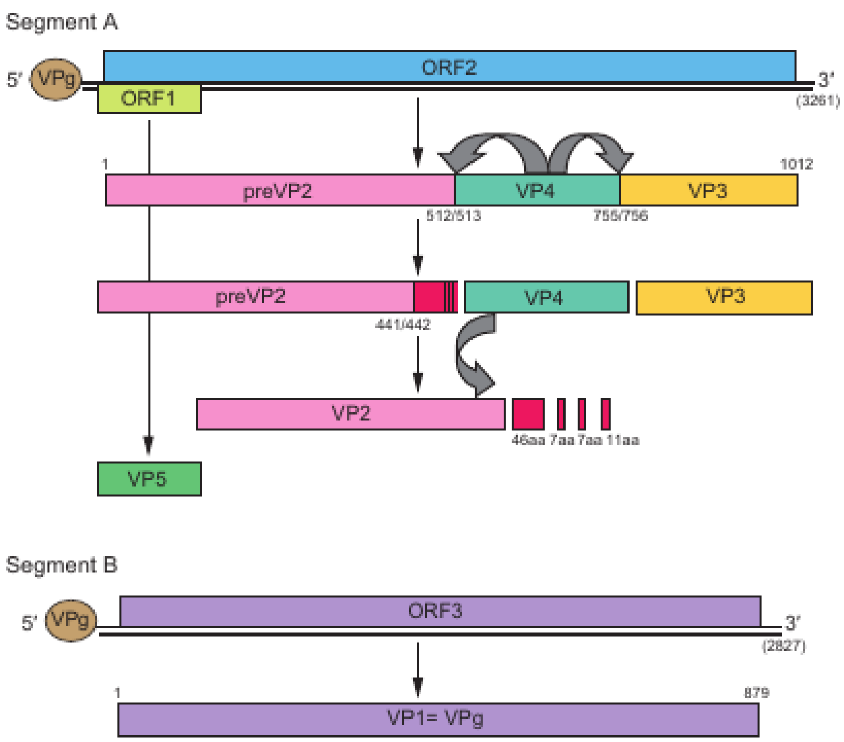

Figura 2: Esquema del genoma de IPNV en el proceso y codificación de proteínas.

En resumen, el IPNV presenta algunas características no encontradas en otros virus de cadena doble de RNA como:

- Su genoma consiste en dos segmentos de cadena doble de RNA; el segmento A es estructural y funcionalmente bicistronico.

- La proteína VP1 se encuentra unida al genoma, VPg y de forma libre dentro de la partícula viral. Los birnavirus son los únicos virus RNA con una unión covalente del RNA viral a la poliproteína.
- El mecanismo de transcripción viral es semiconservativo, en contraste con los reovirus, que tienen transcripción conservativa.

Proteínas virales

El IPNV está formado por 4 proteínas estructurales; VP1, VP2, VP3 VP4 y una no estructural VP5.

VP1: La proteína VP1 está presente en los viriones tanto como un polipéptido libre (94kDa) o como una proteína covalentemente ligada al genoma (106kDa), siendo la proteína de mayor tamaño del virión (Calvert et al. 1991) que representa el 4% de las proteínas de la partícula vírica. Tiene una actividad ARN polimerasa dependiente de ARN, pudiendo participar también en el ensamblaje de la partícula viral (Xu, Si, and Dobos 2004).

Pre-VP2 y VP2: La proteína VP2 es la proteína mayoritaria de la cápsida, de naturaleza antigénica, y corresponde a la región que tiene la mayor variabilidad de todo el genoma. La proteína VP2 es el principal determinante antigénico y de virulencia del virus, contiene los epítopos neutralizantes (Caswell-Reno et al. 1989) y los receptores de unión a las células que determina la especificidad celular y de huésped (Tarrab et al. 1995). Los aminoácidos responsables de virulencia en los serotipos están identificados en una zona hipervariable desde el aminoácido 11 hasta el 30 (residuo 234 al 264). Además, se distingue una zona no variable, entre los aminoácidos 153 y 203 (Frost et al. 1995; Tarrab et al. 1995; Xu et al. 2008).Se han relacionado algunos aminoácidos de la proteína VP2 con la virulencia de los virus, los aminoácidos de las posiciones 217 (Thr), 221 (Ala/Thr), 247 (Thr/Ala) y 500 (Tyr/His). (Blake et al. 2001; Bruslind and Reno 2000; Santi et al. 2004). Los virus de alta a moderada virulencia codifican Ala en la posición 221, mientras que en los aislados de baja virulencia se codifica un Thr en esta misma posición.

VP3: Esta proteína estructural tanto interna como externa del virión interactúa con el material genómico y la proteína VP1 (Hjalmarsson and Everitt 1999; Pedersen, Skjesol, and Jørgensen 2007), ayudando a la organización de la partícula para el ensamblaje (Hjalmarsson, Carlemalm, and Everitt 1999; Luque et al. 2009).

VP4: La proteína VP4 es la proteasa que escinde la poliproteína generada por el ORF mayor del segmento A del genoma vírico, generando las proteínas pre-VP2, y VP3. Se han identificado los puntos de escisión entre los aminoácidos 508 al 509 y entre el 734 al 735.Comparando la secuencia de la proteasa de IPNV se observa que la región C-terminal está muy conservada, pero la región N-terminal incluye una zona variable (Imajoh, Goto, and Oshima 2007; Lee et al. 2007) .

VP5: Es una proteína no estructural rica en arginina codificada por el ORF menor del segmento A del virión (Heppell et al. 1995). Después de la VP2 es la proteína que presenta mayor variabilidad (Suzuki, Kimura, and Kusuda 1998). La función de la proteína VP5 no está clara, se sugiere que puede estar involucrada en apoptosis (Hong, Gong, and Wu 2002). La VP5 parece ser que no es necesaria para la replicación del virus, ni está relacionada de forma directa con su virulencia (Santi et al. 2005) e incluso puede estar ausente en cepas virulentas, aunque se ha visto que los aislados más virulentos suelen tener una proteína VP5 de 12kDa (Santi et al. 2004; Song et al. 2006).

Características físico químicas del virión

El IPNV es muy resistente a las condiciones ambientales. Es termoresistente, se inactiva a 65ºC durante 30 minutos (Roberts 2012). Los viriones pueden permanecer infectivos durante 12 meses a 4ºC, y durante 2 meses a 15ºC (Desautels and MacKelvie 1975). Es resistente a la exposición al éter y cloroformo y sensible al tratamiento con yodo (30 partes por millón (ppm) durante 5 min), al cloro (40 ppm de cloro durante 30 min), al hidróxido de sodio (a pH 12,5 durante 10 min) y también a las radiaciones ultravioleta (Ahne 1982).

Serotipos del IPNV

El IPNV comprende un grupo antigénicamente muy diverso de virus que se han ido aislando y describiendo en los últimos 40 años y que produjo cierta confusión. El primer esquema de agrupación de los aislados propuso tres grandes serotipos denominados VR-299 (serotipo 1) Ab (serotipo 2) y Sp (serotipo 3) (Nicholson and Pochebit 1981; Underwood et al. 1977). Posteriormente, Hill & Way (1995) dividieron los aislados en serogrupos (A y B) (Hill and Way 1995). El serogrupo A comprende nueve serotipos, la mayoría asociados a enfermedad en peces y propusieron la nomenclatura para los serotipos reconocidos hasta entonces como “A1, (arquetipo de West-Buxton más representativo que el VR-299) A2 (o Sp) A3 (Ab) A4 (He) A5 (Tellina o Te), A6 (Can.1) A7 (Can. 2) and A9 (Jasper o JA), mientras el serogrupo B, con menor número de aislados contiene un solo serotipo asociado más a invertebrados marinos. Esta terminología simplificada ha sido ampliamente aceptada. Un esquema se representa en Tabla 1.

En España el serotipo mayoritario detectado es el A2 o Sp, con presencia en algunas zonas, como Galicia, del serotipo A3 (Ab) que es menos virulento (Pérez and Rodríguez 1997; Rodriguez Saint-Jean, Perez Prieto, and Vilas Minondo 1993; Rodriguez Saint-Jean et al. 1994; Rodriguez Saint-Jean, Vilas Minondo, and Perez Prieto 1993).

Tabla 1: Clasificación serológica del IPNV, según Hill and Way 1995 (Hill and Way 1995).

Serogrupo A	Tipo	Familia	Distribución geográfica
A1	AW	*Salmonidae*	América
A2	Sp	*Salmonidae, Cyprinidae*	Europa
A3	Ab	*Salmonidae, Anguillidae*	Europa, Asia
A4	He	*Salmonidae, Cyprinidae*	Europa
A5	Te	*Salmonidae, Ostreidae*	Europa
A6	C1	*Salmonidae*	Canadá
A7	C2	*Salmonidae*	Canadá
A8	C3	*Salmonidae*	Canadá
A9	Jasper	*Salmonidae*	Canadá
Serogrupo B			
B1	TV-1	*Salmonidae,Tellinidae, Ostreidae*	Europa

Replicación viral

El virus replica en el citoplasma celular; el ciclo de replicación dura entre 16 a 20 horas a 22ºC. El IPNV produce un efecto citopático (CPE) característico en los cultivos de células susceptibles con un rendimiento de infectividad de entre 10^6 a 10^9 unidades formadoras de placa (UFP mL^{-1}). Sin embargo, al infectar los cultivos celulares con títulos virales altos, se pueden presentar partículas defectivas (DI), que interfieren en el desarrollo de la infección creando líneas celulares persistentemente infectadas sin evidencias de CPE (Hedrick and Fryer 1981; MacDonald and Kennedy 1979). Hay algunas revisiones que abarcan diversos aspectos de la replicación biológica molecular y características morfológicas del virus IPNV (Dobos 1995; Reno 1999; Rodriguez Saint-Jean et al. 2003).

Respecto a la entrada del virión a nivel celular, se ha observado que la proteína VP2 del virus se une a los polipéptidos de alto peso molecular de la membrana celular (100-200 kDa) que podrían constituir los receptores virales. Esta unión del virión a la célula se satura tras 2-3 horas a 4ºC (Kuznar et al. 1995). La interiorización del virión en las células se realiza por endocitosis (Couve, Kiss, and Kuznar 1992); y se ha demostrado que la multiplicación de virión IPNV es sensible al pH del endosoma, lo cual ayuda a promover la entrada de los viriones hacia el citoplasma (Espinoza and Kuznar 1997; Farias et al. 1988; Kuznar et al. 1995). Por otra parte, se ha propuesto que los virus en general requieren un suministro energético para trasladarse desde la superficie celular al citoplasma. En general, el bajo pH por ejemplo, puede tener una doble función al inducir cambios en la estructura del virión y al proporcionar el gradiente de pH necesario como fuente de energía para la translocación del virión (Carrasco 1994). El ensamblaje de los componentes del virión tiene lugar en el citoplasma de las células infectadas y la liberación del virus se produce por lisis celular. Durante el ensamblaje del virus en el ciclo infectivo, el RNA genómico está encapsulado en una gran partícula de baja densidad (partícula A o pro-virus), inmadura y no infecciosa. La cápsida contiene poliproteínas virales tanto procesadas como no procesadas. Estos pro-virus pueden ser visualizados por microscopía electrónica como partículas abiertas (Figura 3). Después de la maduración, la partícula A es compactada por escisión proteolítica de los precursores virales catalizados por la proteasa viral VP4, formando la partícula B. Las partículas del virus se ensamblan y acumulan en el citoplasma (Villanueva et al. 2004).

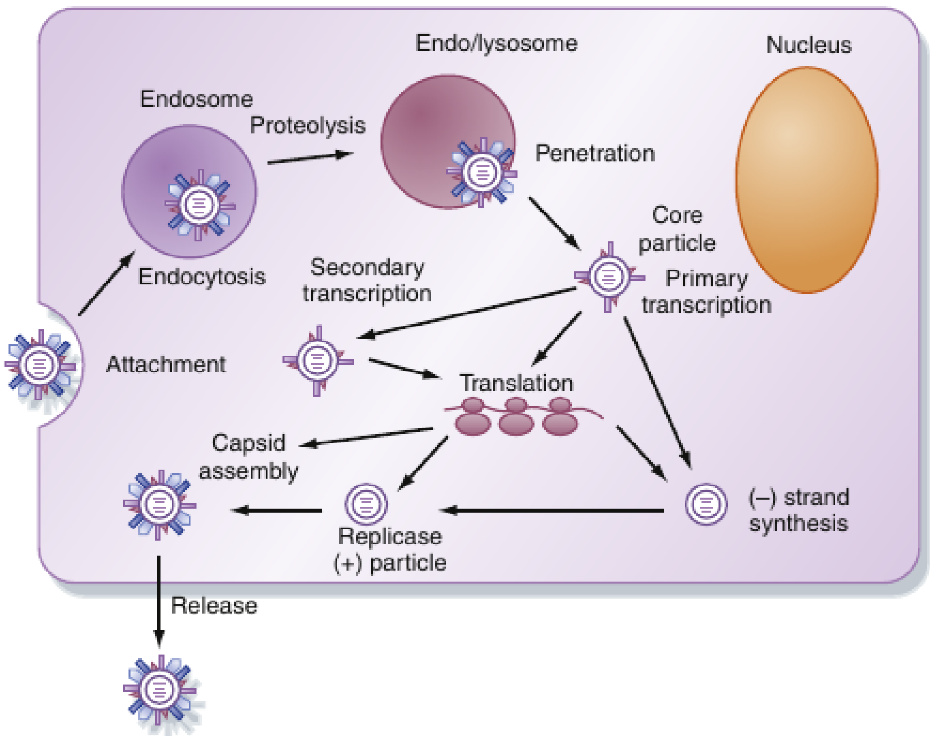

Figura 3: Esquema de infección y replicación en una célula eucariota de un Birnavirus.

Necrosis Hematopoyética Infecciosa (IHN)

En los años 50, paralelamente a los casos descritos de IPN, cuya etiología viral se demostró mucho más tarde (IPNV en los años 60), se detectaron también brotes de otra enfermedad que cursaba con alta mortalidad en juveniles de salmón sockeye (*Oncorhynchus nerka*) en la costa noroeste del Pacífico de Norte América. Como en el caso del IPN, el nombre de la enfermedad describía los síntomas y lesiones más importantes encontradas: enfermedad de la necrosis hematopoyética infecciosa (IHN). En 1958, se aisló el agente etiológico y se mantuvo el nombre del virus de la necrosis hematopoyética infecciosa (IHNV) (Amend 1975).

Esta enfermedad sistémica aguda causa altas mortalidades principalmente en los salmónidos jóvenes. Actualmente es endémica en zonas de la costa del Pacifico de USA y Canadá. Se han descrito brotes epizoóticos en Japón, Corea y algunos países Europeos (Bovo et al. 1987; Enzmann et al. 1992; Hattenberger et al. 1989; Vilas, Rodriguez, and Perez 1994). Esta enfermedad en Europa es de declaración obligatoria ante la Organización Mundial de Sanidad Animal (OIE) (RD, 1614/2008) (OIE 2012).

Los alevines de trucha arco iris son más susceptibles a la enfermedad, especialmente durante los dos primeros meses de vida, con mortalidades cercanas al 80%, mientras que en salmones el periodo más crítico es en peces juveniles, cuando son trasladados al agua de mar, con mortalidades acumulativas de hasta el 90% (Parsons et al. 1986). En trucha, a medida que los peces crecen se crean resistencias ante la infección, sin embargo en concurrencia con otras infecciones, episodios de estrés o alteraciones hormonales, se observan signos de la enfermedad IHNV además del incremento de la mortalidad (Ammayappan, LaPatra, and Vakharia 2010; Liu, Hilleman, and Kurth 1995). La mortalidad es consecuencia de la insuficiencia en el equilibrio osmótico, produciendo edemas y hemorragias. La multiplicación del virus se realiza principalmente en los tejidos hematopoyéticos y células endoteliales de los capilares sanguíneos (Peñaranda et al. 2009; Purcell et al. 2010).

Sintomatología

Los signos clínicos incluyen distensión abdominal, exoftalmia, melanosis y palidez de las branquias. Los peces tienen heces largas semi-transparentes, se encuentran letárgicos con episodios de hiperexcitabilidad. Presentan hemorragias petequiales en la base de las aletas pectorales, boca, músculo cercano al ano y saco vitelino en alevines (Matras, Antychowicz, and Reichert 2006). En alevines menores de dos meses de edad, puede haber pocos signos clínicos a pesar de una alta tasa de mortalidad y los peces supervivientes suelen tener escoliosis (Amend 1975).

Histopatología

En la necropsia se puede observar ascitis con liquido sanguinolento, palidez de los órganos abdominales y petequias viscerales, peritoneales y en el tejido adiposo (Dorson and Torchy 1993). En el análisis histológico se observan hemorragias focales en casi todos los tejidos. En el tejido hematopoyético se evidencian cambios degenerativos celulares, que comienzan con tumefacción, seguido por degeneraciones hidrópicas, núcleos picnóticos, citoplasmas eosinófilos, cariorexis y cariolisis terminando en necrosis. La necrosis de las células granulares eosinófilas del estrato compacto y granuloso intestinal es considerada como un signo patognomónico de la enfermedad. También es característica la dilatación de los senos vasculares renales con abundantes eritrocitos en su luz, así como la presencia de focos necróticos rodeados de linfocitos infiltrados en el hígado y páncreas, además en este último órgano se suelen ver inclusiones intracitoplasmáticas pleomórficas (Kurath et al. 2006).

Patogénesis de la enfermedad

Los brotes de la enfermedad por lo general son más frecuentes en primavera y principios del verano, cuando el agua se encuentra a una temperatura entre 8 a 15°C. Las vías principales de infección son por piel en la base de las aletas (Harmache et al. 2006), branquias y aparato digestivo.

La transmisión del IHNV es horizontal (K. Wolf 1988), también sirven como vectores de transmisión el agua, crustáceos y pájaros (Peters and Neukirch 1986). No se ha demostrado la transmisión vertical, y existe la controversia sobre si el virus permanece persistente en los salmones supervivientes a la infección y se reactiva cuando alcanza la madurez sexual, replicándose tras el estrés ocasionado por el trasvase de los animales, o si los salmones simplemente se infectan durante el proceso de trasvase(Amend and Smith 1975; Bootland and Leong 1999).

Virus de la Necrosis Hematopoyética Infecciosa (IHNV)

Morfología del virión

El virus de la necrosis hematopoyética Infecciosa pertenece a la familia Rhabdoviridae género Novirhabdovirus, tiene una nucleocápsida interna con simetría helicoidal que encierra una molécula de RNA monocatenario de polaridad negativa; posee una envoltura exterior formada por una bicapa lipídica de origen celular en la que se insertan las espículas formadas por la glicoproteína del virión formando la estructura característica de los rhabdovirus, una bala de base plana y punta redonda de 150 a 190 nm de longitud y 65 a 95 nm de diámetro (Chiou et al. 2000) (Ver figura 4).

Genoma viral

El genoma es RNA monocatenario de polaridad negativa que codifica cinco proteínas estructurales: La nucleoproteína (N), fosfoproteína (P), proteína de matriz (M), glicoproteína (G), una RNA polimerasa (L) y una proteína no estructural (non virión o NV) (12 KDa) no descrita en otros rhabdovirus, localizada entre los genes G y L del genoma viral, y cuya presencia es característica del género Novirhabdovirus (Kurath and Leong 1985). Esquema figura 4.

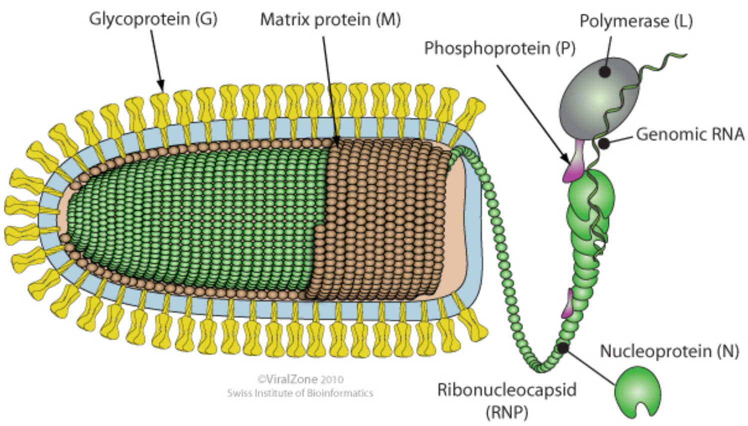

Figura 4: Esquema de un Novirhabdovirus (Fuente: http://viralzone.expasy.org/all_by_species/76.html).

Proteínas virales

Proteína L: Es un componente de la nucleocápsida vírica (220-240 kDa); es la RNA polimerasa RNA dependiente del virión, responsable de la mayoría de las funciones requeridas para la transcripción y replicación.

Proteína G: Esta proteína está asociada en trímeros para formar los peplómeros o espículas de la superficie del virus (monómero 65-90 kDa). La proteína G está implicada en la entrada del virus a las células dianas y en la virulencia del virus en salmónidos. Según la secuencia de la proteína G, el virus se ha clasificado en tres genogrupos U, M y L (Garver, Troyer, and Kurath 2003), aunque los aislados japoneses y coreanos pertenecen a un nuevo grupo (Kim et al. 2007). En trucha arco iris se han identificado diferentes tasas de mortalidad dependiendo del genogrupo viral. El genogrupo M del virus IHNV produce mortalidades elevadas, mientras que con el genogrupo U se han descrito mortalidades bajas (Park et al. 2010). En el salmón rojo (*Oncorhynchus nerka*) los aislados U presentan una alta virulencia, mientras los aislados M tienen baja virulencia (Garver, Batts, and Kurath 2006). La cepa del genotipo U y la cepa M del IHNV son capaces de entrar, replicar y propagarse en tejidos de salmón. Sin embargo, la cepa U lo hace a mayor velocidad que la cepa M. Ambas cepas inducen interferon (IFN) de tipo I, sólo la cepa U es capaz de replicar en presencia de interferón (Purcell et al. 2009). En trucha arco iris, el genogrupo M causa mortalidades más altas que el genogrupo U debido a que la cepa M presenta una mayor tasa de replicación (Peñaranda et al. 2009).

La proteína G contiene epítopos específicos capaces de inducir anticuerpos neutralizantes (Engelking and Leong 1989) y por ello es la candidata ideal en el desarrollo de vacunas para controlar la IHN en salmónidos.

Proteína N: Es el principal componente de la nucleocápsida vírica (47-62 kDa). En la replicación del virus modula el equilibrio entre la transcripción y replicación del genoma al influir en el reconocimiento de las señales de transcripción (Choi et al. 2011).

Proteína P: Es un cofactor de la polimerasa viral (20-30 kDa). La proteína es dimérica y tiene un dominio autónomo plegado central responsable de la dimerización de la proteína (Gerard et al. 2007). Durante el ciclo de replicación, P forma dos complejos diferentes con la proteína N, el complejo N^{0}–P y el complejo de N-ARN-P. Las proteínas N, L y P junto con el RNA del virus forman la ribonucleocápsida (RPN) (Ivanov et al. 2011).

Proteína M: Corresponde a una proteína estructural interna del virión (20-30 kDa). Puede regular la transcripción del genoma de RNA. Esta proteína se encuentra en el núcleo e inhibe la transcripción de la célula huésped, y está involucrada en algunos efectos patológicos como el redondeo de las células infectadas y apoptosis (Chiou et al. 2000).

Características físico químicas del virión

El virus es sensible al pH ácido, formol y éter y resistente al etanol. Es termosensible, puede ser inactivado por secado o calentamiento a 60°C durante 15 minutos (Hill 1975). Es resistente a condiciones medioambientales, el virus puede sobrevivir en agua dulce y salada durante al menos un mes, especialmente en presencia de material orgánico.

Replicación viral

La replicación del virus se produce principalmente en los tejidos hematopoyéticos y células endoteliales de los capilares sanguíneos (Peñaranda et al. 2009). El IHNV infecta a la célula diana por endocitosis a través de la activación de un receptor por la proteína G; el bajo pH del endosoma provoca la fusión entre receptor y la glicoproteína de la envuelta del virus, liberando el complejo ribonucleoproteína (RPN) en el citoplasma. Una vez que la nucleocápsida se libera en el citoplasma, el RNA del genoma se transcribe de forma repetitiva (transcripción primaria) mediante la transcriptasa del virión. La proteína N no se elimina ya que la transcriptasa viral solamente reconoce el complejo RNA-N como plantilla. Para empaquetar los nuevos viriones, las proteínas N, L y P son sintetizadas por los ribosomas libres en el citoplasma celular y se unen al genoma viral de RNA formando la RPN, la cual es asociada a la proteína M produciendo el complejo RPN-M. La proteína G es sintetizada en el retículo endoplásmico (RE), y glicosilada en el aparato de Golgi, antes del transporte y la inserción en la membrana plasmática celular del huésped. El complejo RNP-M migra a las regiones enriquecidas con proteínas G virales de la membrana plasmática para formar la envoltura del rhabdovirus (Wargo, Garver, and Kurath 2010). Ver figura 5.

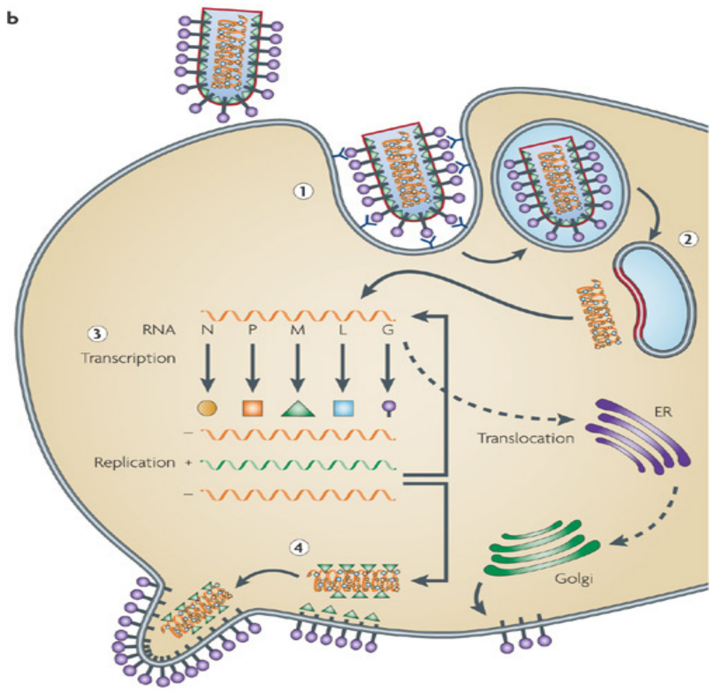

Figura 5: Esquema de infección y replicación en una célula eucariota de un rhabdovirus_ (Schnell et al. 2010).

Prevención y control de las enfermedades virales en acuicultura:

Los peces se crían en condiciones de cultivo intensivo donde la aparición de enfermedades puede originar rápidamente alta mortalidad y graves pérdidas económicas. Las condiciones medio ambientales de las piscifactorías influyen directamente en su estado sanitario y productivo. Para la prevención y control, es importante determinar que los alevines que se comercializan o cambian de una a otra instalación están libres de virus. El aislamiento del virus sobre cultivos celulares para su certificación como libres de IHNV en zonas endémicas de IPNV, requiere establecer pautas o utilizar métodos más sensibles para identificar correctamente el virus.

Medidas zoosanitarias: Las medidas sanitarias tienen como objetivo evitar el contacto del patógeno con el hospedador. Para obtener animales libres de patógenos virales es necesario tomar medidas de control a nivel de las explotaciones, de la región o país y entre los países. A nivel europeo se han establecido leyes de la Comisión Europea como la Directiva 2006/88/CE de 24 de octubre de 2006 del Consejo, relativa a los requisitos zoosanitarios de los animales y de los productos de la acuicultura y a la prevención y el control de determinadas enfermedades de animales acuáticos, con la modificación introducida por la Directiva 2008/53/CE; la lista de terceros países desde los cuales está permitida la importación de determinados animales acuáticos (Reglamento 719/2009/CE), programas de erradicación (Decisión 2009/946/CE), requisitos de cuarentena de los animales de acuicultura (Decisión 2008/946/CE) y directrices para los sistemas de vigilancia zoosanitaria basados en el riesgo (Decisión 2008/896/CE). La normativa nacional básica es el Real Decreto 1614/2008, que es la trasposición de la Directiva Europea 2006/88/CE. Además de los reales decretos como normas a nivel nacional, algunas Comunidades Autónomas tienen sus propias Leyes de pesca (http://www.magrama.gob.es/es/pesca/temas/acuicultura/guia_gestion_sanitaria_acuicultura_tcm7-337018.pdf). Parte del éxito de estos programas depende de la rápida detección e identificación de patógenos específicos. Así, el diagnóstico temprano combinado con estudios epizootiológicos podrían ser utilizados para controlar la enfermedad mediante la prohibición de la comercialización y/o desplazamiento de huevos embrionados infectados o de peces, alevines, adultos y portadores asintomáticos; otras medidas serían la selección de huevos libres de virus y alevines o peces seleccionados genéticamente y resistentes a virus.

Medidas higiénicas: Tienen por objeto evitar que factores medio ambientales alteren, más allá de los límites aceptables, la homeostasis de los peces y predispongan o reactiven enfermedades de origen viral. Los factores a controlar son la calidad del agua, densidad y alimentación.

Medidas terapéuticas: En piscicultura, tradicionalmente se han utilizado como desinfectantes algunos compuestos yodados, cloro, alcoholes y sales de amonio (Amend and Pietsch 1972; K Inouye et al. 1990; Kiyoshi Inouye et al. 1990). Se ha estudiado algunos compuestos con acción antivírica; son sustancias capaces de inhibir o reducir el virus infectivo de la célula huésped. El hecho de que la replicación viral sea absolutamente dependiente de las vías metabólicas esenciales de la célula huésped hace que la búsqueda de agentes antivíricos sea un gran reto y el progreso ha sido bastante lento, con pocos éxitos. El agente antivírico ideal debería inhibir la replicación viral en una fase específica y esencial sin afectar al metabolismo de la célula huésped. Para esto, se deben identificar los sitios diana de infección de los virus, definir sus procesos bioquímicos y las alteraciones que pueden producir en las células infectadas. En la actualidad no hay ningún fármaco con licencia para el control de las infecciones víricas en peces. Sin embargo, se sigue investigando en el tema (Falco et al. 2008; Nácher-Vázquez et al. 2015; Rodriguez Saint-Jean et al. 2013).

Mejora genética: *L*a cría sistemática de peces para aumentar la resistencia natural ante enfermedades infecciosas ha sido estudiada y por ello se han identificado algunos genes marcadores (“quantitative traits locus” QTL) de resistencia frente a IPNV e IHNV (Moen et al. 2009; Ozaki et al. 2001; Verrier et al. 2013).

Inmunoterapia - Vacunación

Entre los retos actuales para disminuir o evitar la dispersión de estos virus, destaca la consecución de vacunas eficaces, económicas, seguras y fáciles de administrar a grandes cantidades de peces (Brudeseth et al. 2013). Las vacunas en acuicultura, además de otorgar protección rápida y de larga duración a individuos con una edad temprana frente a una amplia variedad de serotipos y cepas del virus, deberían evitar la persistencia del virus, puesto que supondría la existencia de animales portadores en la población.

A pesar de la importancia comercial de la acuicultura apenas hay vacunas frente a virus que estén comercializadas. Las vacunas diseñadas no han cumplido con las expectativas ni logrado los efectos beneficiosos esperados, ya que en muchos casos la edad más susceptible a la infección corresponde a peces muy jóvenes (alevines) que no presentan un sistema inmune totalmente desarrollado (Gudding et al. 1997). En el caso de la enfermedad de IPN e IHN en salmones, existen dos momentos críticos para su aparición que corresponden a:

- Alevines menores de 4 meses, y
- Salmón juvenil (smolt) en el momento del trasvase al mar

Para el desarrollo de una vacuna es necesario conocer los mecanismos de defensa del organismo frente al patógeno, conocer la biología del patógeno, identificar los antígenos involucrados en la inducción de una respuesta inmune específica, e implementar ensayos experimentales para determinar la eficacia y seguridad de la vacunación.

Vacunas de DNA

Este trabajo de tesis doctoral se basa en el estudio de vacunas de DNA para los virus IPN e IHN en salmónidos. El desarrollo de las vacunas DNA surge a partir de la observación de que células musculares (miocitos) inyectadas intramuscularmente (IM) con plásmidos de expresión, son capaces de expresar un gen que codifica una determinada proteína de un patógeno (Leong et al. 1997). La inmunización utilizando vacunas de DNA consiste en la transfección de un plásmido de expresión en células eucariotas, dicho plásmido debe codificar el antígeno a través de un gen (Liu 2010). La eficiencia de la inmunización puede variar según el promotor utilizado, La elección del promotor también tiene una profunda influencia en el nivel de expresión del transgen (Williams, Carnes, and Hodgson 2009); existen promotores constitutivos, inducibles o específicos del tejido, como el gen β-actina del músculo o el promotor de la timidina-quinasa. Un promotor inducible, puede ser un promotor regulable con la hormona del crecimiento, secuencias del gen operón lac. El promotor más efectivo por vía IM es del citomegalovirus (CMV), además de ser el más utilizado en estudios de transfección de genes en peces (Heppell et al. 1998). El uso de intrón y señales de poliadenilación (terminación) mejora aún más la expresión (Williams et al. 2009). Sin embargo, estas secuencias reguladoras de origen vírico humanas ocasionan problemas en el momento de legalizar estas vacunas a nivel internacional. Por ello es importante conseguir alternativas como la sustitución de los promotores víricos por promotores específicos de tejido de origen piscícola; algunos resultados han sido descritos con el gen IRF1A en la trucha arco iris o con otros genes (Alonso et al. 2003; Ruiz et al.

2008). La inmunización genética con DNA ofrece muchas ventajas frente a otros métodos de inmunización tales como vacunas de virus inactivado, atenuado o recombinantes.

Entre ellas:

* Mimetiza la infección viral con la expresión y síntesis de una sola proteína en la célula inyectada, obteniendo una proteína correctamente plegada. De esta manera es semejante a las vacunas vivas atenuadas, pero sin el riesgo de reversión de la infección (N Lorenzen et al. 2002; Purcell et al. 2006).

* Induce una respuesta inmune innata no específica. Se ha demostrado la expresión de genes inducidos por interferón (IFN, Mx, Vigs).

* Estimula la producción de anticuerpos específicos para el antígeno expresado. Las vacunas de DNA inducen los tres brazos de la inmunidad adaptativa, es decir; células T auxiliares, células T citotóxicas y anticuerpos, generando una respuesta inmune frente el antígeno expresado *in-vivo* (Holvold, Myhr, and Dalmo 2014).

* Produce la expresión continuada del antígeno, no se necesitan inmunizaciones de recuerdo a corto plazo (Kurath, Purcell, and Garver 2007; N. Lorenzen et al. 2002).

*Son muy seguras, debido a que no hay reversión del virus (N Lorenzen et al. 2002; Purcell et al. 2006).

* Se pueden modificar los genes que codifican los antígenos vacunales por mutagénesis dirigida y así aumentar su eficacia (Lorenzen and LaPatra 2005).

* Se puede incorporar más de un gen de un patógeno o genes de diferentes patógenos y/o coadyuvantes diferentes a los ya incluidos en el plásmido como los motivos CpG (Plant and Lapatra 2011).

* Bajo coste económico, fácil de fabricar y no requieren de una cadena de frío para su almacenamiento y transporte (Kurath 2005).

Distribución de las vacunas DNA en peces

El plásmido administrado por vía I.M. puede transportarse por vía sanguínea, sin embargo, en este proceso parte del plásmido puede ser degradado por endonucleasas (Tonheim, Bøgwald, and Dalmo 2008). A pesar de esto, el plásmido intacto se ha observado en órganos internos como hígado, bazo, riñón anterior, corazón e intestino. Sin embargo, la mayor cantidad de plásmido se encuentra en el sitio de la inyección. La captación del plásmido a nivel celular se realiza por endocitosis y pinocitosis (Traxler et al. 1999). Los miocitos y células mononucleares son capaces de captar rápidamente el pDNA. (Tonheim, Bøgwald, et al. 2008). Se sugiere que la inyección produce poros e interrupciones en las membranas de los miocitos permitiendo la transfección del DNA (Heppell et al. 1998); otros estudios describen receptores de reconocimiento de patrones que podrían reconocer y unirse a los plásmidos (Desmet and Ishii 2012).

Métodos de administración de vacunas DNA

Las vacunas DNA se desarrollaron como un método de vacunación por vía intramuscular, el DNA era capaz de transfectar celular musculares. En estos últimos años, los métodos se han ido perfeccionando para adaptarlos a los tamaños de los peces y condiciones de los cultivos (Anderson, Mourich, and Leong 1996; S. Corbeil et al. 2000).

Tabla 2: Microcápsulas de administración de vacunas de DNA oral en acuicultura.

Partículas	Recubrimiento	Proceso de recubrimiento	Especie y referencia
Alginato	ß(1,4)-D-manurónico y residuos de ácido α(1,4)-L-glucurónico	Encapsulación	Lenguado japonés (J.-Y. Y. Tian, Sun, and Chen 2008)
Quitosano	ß(1,4)-D-glucosamina y copolimero N-acetil-D-glucosamina	Encapsulación	Tilapia del Nilo *(Oreochromis niloticus)* (Leal et al. 2010); Lenguado japonés (J. Tian, Yu, and Sun 2008) y Lubina asiática (*Lates calcifer*) (Kumar et al. 2008)
PLGA	ácido poli(D,L-láctico–co–glicólico)	Encapsulación	Trucha arco iris (Adomako et al. 2012), Salmon Atlántico (Munangandu et al. 2012; Nielsen, Fredriksen, and Myhr 2011).
Liposomas	Vesículas artificiales lipídica bicapas	Encapsulación	Rodaballo (Harikrishnan et al. 2012; Leon-Rodriguez et al. 2013)
Polycaprolactona	Polímero sintético biodegradable	Recubrimiento	Carpa de la india (*Labeo rohito*) (Behera and Swain 2012)
Fosfato de calcio	Material inorgánico, biodegradable y biocompatible	Recubrimiento	Carpa de la india (Behera and Swain 2012).

La vacunación oral es uno de los métodos que proporciona mayores ventajas para la vacunación masiva de poblaciones de peces, ya que no implica una manipulación directa del animal y se pueden vacunar alevines sin generar lesiones. Los plásmidos deben estar protegidos de la inactivación o digestión durante su paso por el tracto gastrointestinal. Se han ensayado microesferas con diferentes materiales (Ver tabla 2) como alginato de sodio (de las Heras, Rodríguez Saint-Jean, and Pérez-Prieto 2010) chitosan y algunos polímeros como PLGA (poly(lactic-*co*-glycolic acid)) (MacLaughlin et al. 1998; J. Tian, Sun, et al. 2008).

Respuesta Inmune de peces: Vacunas DNA

El gran desarrollo de la acuicultura durante los últimos años ha despertado el interés por el sistema inmune de los peces con el fin de poder establecer estrategias adecuadas para controlar las enfermedades infecciosas mediante vacunas. Los peces teleósteos son, en términos evolutivos, el grupo de vertebrados más primitivo con un sistema inmune completo, con respuesta inmune innata y respuesta inmune específica o adquirida frente a un patógeno.

Respuesta Inmune innata

La inmunidad innata o los mecanismos de defensa inespecíficos constituyen la principal línea de defensa en peces teleósteos y juega un importante papel en la protección frente a patógenos invasores (Gadan et al. 2012). principalmente porque actúa de manera rápida e independiente de la temperatura (Ellis 2001). El conocimiento de la inmunidad innata en teleósteos es aún limitado, aunque en los últimos años, se ha realizado numerosos avances y estudios a nivel molecular, identificando y determinando las secuencias de muchas citoquinas. Las citoquinas son proteínas secretadas por células del sistema inmune y que regulan la respuesta frente a patógenos. La actividad de las citoquinas se intuyó en peces en 1960 (Callard and Gearing 1994). Posteriormente, se han identificado y caracterizado varias isoformas de IFNs e interleuquinas (ILs) como la IL1β, IL6 (Peddie et al. 2001), IL8 (Laing et al. 2002), IL10, IL12 y factor de Necrosis Tumoral (TNFs) (Laing et al. 2001). También se han identificado varias quimioquinas (K J Laing and Secombes 2004).

Interferones:

Los interferones (IFNs) son citoquinas inducidas por virus, que comparten propiedades antivíricas. La producción de interferón es uno de mecanismos de defensa inespecífico más importante frente a las infecciones virales. De las tres familias de IFNs conocidas en vertebrados superiores, dos se han descrito como importantes para la defensa antivírica en peces Teleósteos. La familia de los IFNs del tipo I agrupa dos subfamilias, que posiblemente interactúan con diferentes receptores; los IFNs del tipo II constan de dos miembros el IFN tipo γ, con funciones similares al IFN- γ de mamíferos, y un IFN-γ relacionado (IFNγ rel) que es específico de teleósteos y cuyas funciones aún no son bien conocidas (Zou and Secombes 2011). El interferón tipo I induce un estado antiviral en células que impide la propagación del virus. Este estado se produce gracias a la inducción de proteínas que actúan como mediadores intracelulares. Los virus de salmónidos son inductores de interferón y de las proteínas antivíricas IFN-dependientes como la Mx, Vigs, ISG, etc.

La proteína Mx interfiere en la replicación viral al inhibir la actividad transcripcional sobre complejos ribonucleoproteicos; éstas proteínas son inducidas por el IFN en las primeras etapas de la infección por lo cual se ha utilizado como marcador de la actividad de IFN en teleósteos (Trobridge and Leong 1995). En general, la inducción de la expresión del gen Mx es una respuesta inmune innata rápida y bastante eficaz frente a una infección viral durante un periodo de tiempo suficiente para permitir que la respuesta inmune específica se desarrolle (Leong et al. 1998). Se ha demostrado la relación entre la expresión de genes de IFN-γ con el nivel de resistencia de trucha arco iris al virus IHNV (Kurath et al. 2010). También se sugiere que el sistema de IFN podría estar implicado en la persistencia viral y contribuir a la formación de portadores asintomáticos del IPNV (Gadan et al. 2013; Polinski et al. 2010). Las proteínas Vigs, Vig1 y Vig2; la proteína Vig1, fue descubierta por primera vez en trucha arco iris infectada con VHSV (Boudinot et al. 1999), más tarde fue encontrada en humanos y renombrada como viperina (proteína inhibidora de virus, asociada al retículo endoplasmático, inducible por IFN).

Respecto a las interleuquinas, la interleuquina 8 (IL8) en salmónidos es producida principalmente por macrófagos y es quimiotáctica de neutrófilos, monocitos y eosinófilos. Participa junto con otras interleuquinas como la il1b y el TNF en la respuesta inflamatoria; aunque no está claro cómo la inflamación interactúa con la respuesta de interferón aparentemente correlacionadas (Tafalla, Rodriguez Saint-Jean, and Pérez-Prieto 2006). Por lo cual, la interacción entre la inflamación y la respuesta a IFN de tipo I merece más atención ya que estos dos mecanismos son críticos para el éxito en el control de infecciones virales y/o el desarrollo de la respuesta adaptativa.

Quimioquinas:

Son una familia multifuncional de citoquinas que presentan actividad quimiotáctica promoviendo la movilización leucocitaria hacia el sitio de infección y regulando la respuesta y diferenciación de las células reclutadas. Se caracterizan por la presencia de cuatro residuos de cistina y se dividen en cuatro subfamilias en función de la posición de las dos primeras cisteínas en su secuencia: CXC (a alfa), CC (b beta), C y CX_3C (Wiens et al. 2006); como se puede observar en la figura 6:

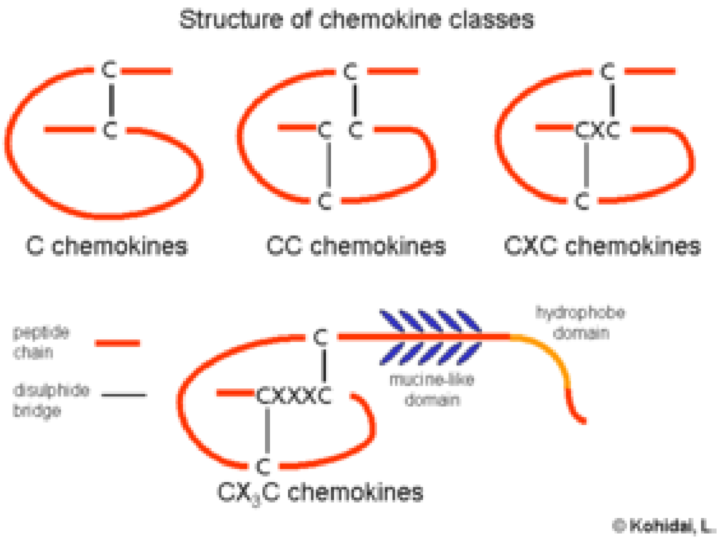

Figura 6: Esquema con los diferentes tipos de quimioquinas.

En condiciones fisiológicas normales, las quimioquinas están implicadas en la segregación y maduración de las células linfocitarias, y han sido relacionadas con otros procesos como el desarrollo neuronal o de organogénesis. Sin embargo es cada vez más evidente que la mayoría de las quimioquinas tienen una función doble, es decir, que aunque cumplen unas funciones fisiológicas en condiciones normales, se inducen en mayor cantidad frente a una infección para condicionar la respuesta inmune inducida. El primer gen de quimioquina identificado en un teleósteo fue en trucha arco iris, en 1998 y se denominó CK1. Desde entonces se han identificado numerosos y variados genes de quimioquinas en diversas especies de peces pero su papel en homeostasis y respuesta inmune continúa siendo desconocido en muchos aspectos (Alejo and Tafalla 2011). Las quimioquinas que se estudiaron en el trabajo de tesis son:

CCR7: El receptor quimioquinas C-C de tipo 7 es una proteína codificada por este gen “CCR7”, que es miembro de la familia de receptores de unión de la proteína G. Este receptor en mamíferos fue identificado como un gen inducido por el virus de Epstein-Barr (VEB), con efectos sobre los linfocitos B. Este receptor se expresa en diversos tejidos linfoides y activa linfocitos B y T. Puede estar involucrado en el control de la migración de las células T de memoria a los órganos linfoides secundarios, tales como los ganglios linfáticos, así como estimular la maduración de células dendríticas en mamíferos (Eo et al. 2001; Ordás et al. 2012).

CCR9: Receptor de la quimioquina de tipo C-C 9. La proteína codificada por este gen es un miembro de la familia de receptores de quimioquinas beta. Es una proteína de transmembrana similar a los receptores acoplados a la proteína G. Las quimioquinas y sus receptores son los principales reguladores de la migración y maduración de timocitos en condiciones normales y la inflamación en mamíferos. Se ha descubierto que este gen se expresa diferencialmente por los linfocitos T en el intestino delgado y colon, sugiriendo un papel en el reclutamiento y desarrollo de timocitos que pueden permitir la especialización funcional de las respuestas inmunes en diferentes segmentos del tracto gastrointestinal (Richmond 2008). Las siguientes quimioquinas, se encuentran dentro del grupo de las quimioquinas CCL27/28 y CCL19/21/25. Pertenecen a los mismos grupos a los que se asocian las quimioquinas de mayor importancia en la respuesta inmune de mucosas en mamíferos (Akuthota et al. 2013). Por lo tanto, las quimioquinas de trucha arco-iris tienen una homología filogenética similar y funcionalidad, por lo cual pueden ser catalogadas como ortólogos.

CK9: Puede estar relacionada con el aumento de la capacidad quimiotáctica en las células epidérmicas y/o endoteliales en repuesta a una infección (Montero et al. 2009).

CK10,CK11 y CK12: Tienen un perfil transcripcional en truchas no tratadas similar a una quimioquina asociada claramente a una respuesta de tejidos linfoides periféricos (Montero et al. 2009). Resulta interesante por el hecho de presentar una regulación en tejidos mucosos durante infecciones virales con VHS.

Respuesta Inmune adquirida o específica

La inmunidad específica o adquirida es más rudimentaria en peces teleósteos que en mamíferos; solo hay tres isotipos de inmunoglobulinas y algunos peces carecen del complejo mayor de histocompatibilidad (MHC). La respuesta inmune adquirida frente a virus, aunque es específica y tiene memoria, es más lenta respecto a la repuesta innata, y además es dependiente de la temperatura (Ellis 2001). Los peces vacunados desarrollan una respuesta específica frente al patógeno transcurridos de 15 a 30 días, dependiendo del tamaño de los peces y dosis de la vacuna (McLauchlan et al. 2003). La respuesta específica de los peces tras la vacunación puede ser tanto humoral como celular.

Respuesta humoral:

En peces teleósteos, se asumía la existencia de un único tipo de inmunoglobulinas (Ig) similar a la IgM de mamífero aunque tetramérica. Posteriormente se han identificado tres isotipos: IgM, (Hordvik et al. 2002); IgT e IgD (Salinas, Zhang, and Sunyer 2011). La IgT ha sido descrita en trucha (Hansen D. et al. 2005), también denominada IgZ en el pez cebra; y ha sido identificada como una Ig especializada en mucosas (Hansen D. et al. 2005; Hatten et al. 2001). Hasta el momento, sólo tres isotipos de Ig se han caracterizado en los linfocitos B de salmónidos, IgM, IgD e IgT.

Ig M: Corresponde al isotipo más abundante en suero, alrededor de 1.000 veces más que los niveles de IgT en peces. Los anticuerpos confieren inmunidad protectora contra las infecciones sistémicas, por lo cual esta Ig es bastante utilizada en los estudios de respuesta a la vacunación, la IgM se ha detectado también en las superficies mucosas de la piel, intestino y branquias (Kaattari, Evans, and Klemer 1998).

Ig D: El reciente descubrimiento de la forma secretora de la IgD caracterizada en la trucha arco iris muestra que este isotipo no sólo existe unida a la membrana de Ig, sino que además se ha detectado en suero. Las células plasmáticas secretoras de IgD en la trucha arco iris fueron más altas en el riñón y bazo. La relación de IgM e IgD en branquias es similar, lo que indica un papel protector de esta inmunoglobulina en mucosas (Salinas et al. 2011).

Ig T: Su descubrimiento, caracterización y clonación en la trucha arco iris ha sido esencial para implicarla principalmente en la protección de mucosas y como tal, los niveles de IgT detectados en tejido de mucosas es alrededor de 100 veces más altos que los detectados en el suero (Hansen D. et al. 2005; Zhang et al. 2010).

Respuesta celular:

Es una forma de respuesta inmunitaria de selección natural mediada por linfocitos T. Actúa como mecanismo de ataque contra microorganismos intracelulares, como los virus, que son capaces de sobrevivir y proliferar en el interior de los fagocitos y otras células del huésped, por lo cual no son neutralizados por los anticuerpos circulantes. Por esto, esta respuesta induce la destrucción del microorganismo residente en los fagocitos o de las células infectadas mediante los receptores de células T (TCR) que puede reconocer antígenos presentados por el complejo mayor de histocompatibilidad MHC-I o MHC-II, estimulando a los linfocitos T $CD8^+$ (células T citotóxicas) y linfocitos T $CD4^+$ (células T helper o colaboradoras) respectivamente. La respuesta inmune celular está representada por la activación de los linfocitos Th1, que secretan citoquinas pro-inflamatorias, y las células T $CD8^+$ que pueden matar o eliminar a las células presentadoras del antígeno.

En el caso de la administración de vacunas DNA, se ha documentado que la respuesta inmune es llevada a cabo principalmente por las células presentadoras de antígeno (APC) en mamíferos, en los cuales por ejemplo, las células dendríticas (DC) y las APC profesionales, tales como los macrófagos contienen pDNA después de la administración IM (N Lorenzen et al. 2002). Las APC en el sitio de administración atraen a las células de respuesta inmune como linfocitos T para la presentación de antígenos. Las APC contienen en el citoplasma el pDNA que puede expresarse en ésta célula produciendo proteínas inmunogénicas e imitando una infección de un patógeno intracelular, permitiendo la presentación del antígeno al MHC-I sobre la superficie de la APC. Por otra parte, las células presentadoras de antígenos también pueden captar antígenos solubles liberados de otra célula transgénica (por ejemplo, un miocito), procesando y presentando el péptido en la superficie celular ante el MHC-II. El TCR puede reconocer estos péptidos presentados tanto por el MHC-I y MHC-II, estimulando a los linfocitos T CD8 (células T citotóxicas) y linfocitos T CD4 respectivamente. Además, las APC también pueden presentar antígenos producidos por células en apoptosis de los miocitos transfectados (u otras células) (Sommerset et al. 2003). Una vez se reconozca el antígeno por el TCR del MHC-I o MHC-II, es el linfocito B (células secretoras de anticuerpos) la célula que reconocerá al antígeno a través de sus receptores. Una de las características principales de las vacunas de DNA es su capacidad de estimular tanto el sistema inmune celular, incluyendo la citotoxicidad, como la respuesta inmune humoral (Kurath et al. 2007).

En la tabla 3, se muestra la comparación del sistema inmune adaptativo basado en las inmunoglobulinas de teleósteos y mamíferos.

Tabla 3: Comparación de los elementos claves del sistema inmune adaptativo basado en las inmunoglobulinas de peces teleósteos y mamíferos (Sunyer 2013).

	Teleósteos	Mamíferos
Inmunoglobulinas	IgM, IgD e IgT (IgZ)	IgM, IgG, IgA, IgD e IgE
AID	Sí	Sí
Cambio de Clase-recombinación	No	Sí
Hipermutación somática	+++	+++
Maduración de la afinidad	+	+++
Respuestas de memoria	+	+++
TCR, CD4, CD8	Sí	Sí
MHC de clase I y II	Sí	Sí
CD28, CD40, CD80, CD86, ICOS	Sí	Sí
Th1, Th2 y Th17 citoquinas	Sí	Sí
Bazo, el timo y médula ósea	Sí	Sí
Tejido linfoide asociado a mucosa	El bazo y el timo, pero hay verdadera médula ósea	Sí
Centros germinales y ganglios linfáticos	No	Sí

Células B

En mamíferos y otros vertebrados incluyendo a los peces, la fagocitosis es llevada a cabo principalmente por las células polimorfonucleares, monocitos y macrófagos (Neumann et al. n.d.; Rabinovitch 1995). En los últimos años se ha descubierto que las células B IgM^+ primarias de los teleósteos tienen una potente capacidad fagocítica (Li et al. 2006; Øverland et al. 2010). Estas células han sido descritas como células B-1. La fagocitosis se produce de una manera independiente del receptor de antígeno de células B. Ambos subconjuntos de células B-1 fagocíticas (B-1a y B-1b) son capaces de madurar sus fagosomas en fagolisosomas y capaces de lisar bacterias internalizadas. Además de tomar partículas grandes, las células B-1a y B-1b en la cavidad peritoneal son capaces de presentar antígenos a partir de partículas internalizadas a las células T $CD4^+$ (Parra et al. 2012)- Sin embargo no se conoce si estas células pueden presentar antígenos a las células T $CD8^+$.

Inmunidad de mucosas en peces:

En peces teleósteos, las barreras de protección frente a patógenos del medio ambiente son zonas de tejido linfoide (MALT) en mucosas y se les denomina Inmunidad de mucosa. En los últimos años, se han publicado revisiones (Salinas et al. 2011) y trabajos relacionados con el tema. Se trata de un sistema aún no bien conocido en teleósteos, y que teóricamente podría tener una significación especial en sistemas de vacunación oral. Sin embargo hay pocos estudios de respuesta inmune antiviral en vacunas administradas por vía oral (Sato and Okamoto 2010). Algunos autores, reconocen la presencia de un sistema inmune integrado que comunica todos los sitios inductores y efectores del sistema inmune de las mucosas, denominado Sistema inmune mucoso común (CMIS) (Iijima, Takahashi, and Kiyono 2001); sin embargo, otros autores consideran al CMIS obsoleto, y atribuyen a las quimioquinas y sus receptores, una respuesta integrada en los tejidos de mucosas.

El sistema inmune de mucosa presenta una amplia regionalización y compartimentación (Brandtzaeg 2010; Macpherson et al. 2008), dificultando las estrategias eficaces de vacunación, como ha sido observado en la inmunización nasal en mamíferos y su respuesta en el GALT (intestino, tracto respiratorio y urogenital) (Chen and Cerutti 2010). Estas asociaciones son menos claras en el caso de los teleósteos, por lo cual una pregunta clave es ¿podemos inmunizar peces por una vía mucosa e inducir protección en todos los sitios de la mucosa? (Salinas et al. 2011).

En carpa infectada con *V. anguillarum* por vía anal se han observado respuestas específicas de IgM en el intestino, piel, bilis y suero; mientras que en la infección por vía oral, no se detectó la inducción de respuestas en suero. En tilapias inmunizadas con HGG por vía oral y anal, se observaron respuestas específicas de IgM en plasma, bilis y moco cutáneo (Jenkins 1994). Una de las áreas más importantes para investigar a fin de abordar la presencia de un CMIS en los teleósteos, es el nicho específico “homing” de las células B y T en los órganos linfoides sistémicos y de mucosa durante el curso de la infección o el desarrollo de una respuesta inmune. Para ello, es fundamental dilucidar la participación de quimioquinas y sus receptores en los linfocitos de mucosas y sitios sistémicos (Alejo and Tafalla 2011). Hasta el momento, se sabe muy poco sobre el homing de las células T y B en los órganos linfoides de los teleósteos, un estudio ha demostrado que los linfocitos T intraepiteliales del intestino en trucha parecen ser fenotípicamente y funcionalmente idénticos a las células T sistémicas (Bernard et al. 2006). En mamíferos y aves, la principal inmunoglobulina del tejido de mucosas corresponde a la IgA (considerada como una forma primitiva de inmunidad adaptativa que regula las poblaciones microbiales del intestino (Macpherson et al. 2000; Suzuki et al. 2007), y en los anfibios corresponde a la IgX (Flajnik and Kasahara 2010).

En teleósteos, la IgM es la única inmunoglobulina encontrada en la respuesta sistémica y la IgT ha sido categorizada como la inmunoglobulina especializada en la inmunidad de mucosas en intestino (Zhang et al. 2010). La inmunidad de mucosa o tejido linfoide de mucosa (MALT) contiene una variedad de leucocitos e inmunoglobulinas (Ig) (Brandtzaeg 2009) y se encuentra en: 1. El tejido linfoide del intestino (GALT); 2. El tejido linfoide de la piel (SALT) y 3. El tejido linfoide de las branquias y zona interbranquial (GIALT).

En intestino, las células linfoides se encuentran de forma dispersa. Sin embargo es posible identificar una zona rica en macrófagos, granulocitos, linfocitos y células plasmáticas en la lámina propia (LP); y otra zona extraepitelial (IEL) con células T y algunas células B. En la tabla 4, se observan las principales características de este tejido (GALT) en mamíferos y teleósteos. Ver tabla 4.

En las branquias y zona inter-branquial (GIALT), existen leucocitos dispersos en el epitelio de las laminillas y organizadas en áreas dentro del arco branquial. En el mucus de la branquias se ha detectado IgM (Ferguson et al. 1992).

Tabla 4: Características fundamentales entre el GALP de mamíferos y teleósteos (Sunyer 2013).

	Teleósteos	**Mamíferos**
Principales inmunoglobulinas en GALT	IgT polimérica	IgA polimérica
Principales subconjuntos de células B en GALT	Células B IgT^+	Células B1 y B2 productoras de IgA
Ultraestructura GALT	LP, IELs, No PP y MLNs	LP, IELs, PP, MLNs
Principales inmunoglobulinas en intestino	IgT	IgA
pIgR como transporte de sLg al lumen intestinal	Sí	Sí
Respuesta especifica de IgT o IgA frente a parásitos intestinales	Sí	Sí

Aspectos de regulación para las vacunas de DNA:

En Europa hasta ahora no se ha aprobado ninguna vacuna DNA en acuicultura. Sin embargo, la Agencia de Inspección Alimentaria de Canadá (CFIA) ha aprobado una vacuna de DNA frente a IHNV para uso comercial en Canadá en 2005 (Salonius et al. 2007) que lleva varios años administrándose sin problemas, y es esperable que en un futuro se extienda su uso.

Vacunas de DNA para IPNV

Desde el aislamiento del IPNV se han desarrollado diferentes estrategias para la elaboración de vacunas contra el virus, debido al gran interés a nivel comercial de este virus para las industrias acuícolas, existen diversas vacunas y estudios realizados por empresas farmacéuticas. Sin embargo, la documentación sobre estas vacunas en revistas científicas es bastante escasa. Las vacunas comerciales disponibles sueles ser vacunas polivalentes que contienen el virus completo inactivado o vacunas de subunidades basadas en la proteína recombinante VP2 (VP2r) del virus producida en bacterias.

En Chile se han registrado varios productos indicados para IPNV, en su mayoría vacunas polivalentes (frente a diferentes patógenos) e inyectables. De las más de treinta vacunas registradas, ocho son exclusivas para IPNV, una de ellas es una vacuna oral (Aquavac IPN Oral, Shering Plough Cia. Ltda) http://webdesa.sag.gob.cl/sites/default/files/lista_salmonidos_registro_provisional_12-5-2014.pdf. En Noruega también se vacuna frente al IPNV con vacunas recombinantes, una de ellas es la vacuna polivalente en la que la VP2r de IPNV se añadió a una vacuna existe frente a furunculosis y vibriosis (Norvax R Protect-IPN, NP-IPN) (Frost and Ness 1997).

En el caso de IPNV, debido a la eficacia controvertida de las vacunas tradicionales de virus inactivados y las vacunas de subunidades, la búsqueda actual de alternativas para la lucha contra este virus se centra en vacunas de DNA codificando para genes de la poliproteína o la VP2 (de Las Heras, Pérez Prieto, and Rodríguez Saint-Jean 2009; de las Heras et al. 2010). En el Laboratorio de Virus de Peces del CIB, se desarrolló y caracterizó una vacuna DNA frente al IPNV administrada por vía oral. En esos trabajos se demostró que la construcción pcDNA- VP2 era capaz de expresar la proteína VP2r del virus IPN en diferentes tejidos de los animales inoculados con el plásmido de expresión. Se comprobó también la capacidad de la vacuna para estimular respuesta inmune innata y para inducir anticuerpos neutralizantes específicos frente al virus. Los ensayos de protección con la vacuna pcDNA-VP2 administrada por via oral dieron buenos resultados, RPS de 66 a 94 % dependiendo de edad de los peces e infectividad del virus (de las Heras et al. 2010).

Vacunas de DNA para IHNV

En 1996 el grupo de J. Leong del laboratorio de la Universidad del Estado de Oregón desarrolló una vacuna frente al IHNV que, en experimentos de laboratorio, fue mucho más efectiva respecto a la vacuna de virus inactivado o de subunidades utilizadas previamente (Anderson, Mourich, Fahrenkrug, et al. 1996).

La vacuna consistía en un plásmido vector que codificaba el gen de la glicoproteína de superficie (G) bajo el control transcripcional de un promotor de CMV; esta vacuna DNA se diseñó para expresar únicamente la proteína G del virus porque estudios previos habían señalado que esta proteína purificada era la única proteína viral capaz de inducir anticuerpos neutralizantes frente al IHNV (Engelking and Leong 1989). Su administración intramuscular provocó la expresión de la proteína G del IHNV en el músculo, y la inducción de una respuesta antivírica temprana e inespecífica mediada por IFN en los peces vacunados y más tarde una respuesta inmune específica y prolongada; registrando niveles altos de protección frente al IHNV (83% al 96% de los peces vacunados respecto a un 10% al 15% de los peces no vacunados). Hay que resaltar que, de las vacunas DNA ensayadas en varias especies animales y en humanos, las vacunas DNA frente a rabdovirus en peces han dado los resultados más prometedores. En 1977 se patentó una vacuna DNA frente al virus IHN, que contenía el promotor del citomegalovirus CMV (Heppell and Davis 2000). Posteriormente, la Agencia Canadiense de inspección alimentaria (División Veterinaria, Biológica y Biotecnológica) aprobó en Canadá una vacuna de similares características que ha sido comercializada por Novartis (Apex-IHN) y constituye la primera vacuna DNA comercial utilizada en acuicultura.

Las vacunas DNA son muy efectivas induciendo protección mediante respuesta inmune celular y humoral en peces, como se ha mencionado anteriormente. Sin embargo existen algunos inconvenientes porque, puesto que los peces cultivados se destinan a la alimentación, su uso está limitado por una serie de restricciones legales tales como las que se refieren al origen de los elementos reguladores que controlan la expresión del antígeno.

Desde 1993, la mayoría de las vacunas DNA contienen el CMV como promotor porque es potente y dirige la transcripción en una gran variedad de tipos de células eucarióticas. Sin embargo, puesto que procede de un virus patógeno humano, se ha considerado “no seguro” por las agencias federales que licencian los procesos de vacunación genética en peces destinados al consumo humano. Así en Estados Unidos la FDA (Food and Drug Administration, USA) ha propuesto que el vector de expresión debería ser de origen eucariótico, preferiblemente de peces (Food, Drug Administration (FDA), and Research 2007).

De acuerdo con esta idea el grupo de la Dra. JA Leong en Oregon diseñó un plásmido de expresión que contiene un promotor específico de trucha que dirige la expresión de la proteína G del IHNV y que sustituye al habitual promotor CMV humano; el promotor es el “Interferon Regulatory Factor 1A" (IRF1A) de trucha arco iris, cuya eficacia en la inducción de repuesta inmune frente al IHNV fue evaluada en alevines de trucha arco iris inyectados intramuscularmente. En las mismas condiciones experimentales, la protección lograda fue mayor que la obtenida con el vector tradicional (Alonso et al. 2003). Esta vacuna DNA (IRF1A-G) ha sido la seleccionada para la realización de los experimentos de vacunación oral frente a IHNV en este trabajo de Tesis. Además de la preocupación que despierta el uso de determinados vectores de expresión con promotores de virus humanos, otro de los problemas que surgen en el uso de las vacunas DNA es su seguridad en aspectos medioambientales, con el posible desarrollo de tolerancia o autoinmunidad y el potencial de integración de la vacuna DNA en el genoma del animal vacunado. Sin embargo, tras casi 15 años de utilización de la vacuna comercial para IHNV, no se ha demostrado que haya sucedido ninguno de estos problemas.

No obstante, la investigación dirigida a subsanar estas dudas y problemas para el uso de estas potentes vacunas ha proseguido. Fruto de ello ha sido el diseño de una original "vacuna suicida" frente al IHNV que provoca la destrucción del plásmido vector después de que los módulos de la vacuna se han expresado (Alonso, Chiou, and Leong 2011). La vacuna suicida incorpora un gen M de la matriz del virus, capaz de dirigir la muerte programada de las células. La vacuna tiene dos operones: Induce primero una respuesta inmune para la proteína G y después conduce a la célula hacia la apoptosis para eliminar las células que contengan la vacuna DNA.

Estas investigaciones indican que la búsqueda de soluciones para la utilización de vacunas DNA es un proceso continuo. Mejorar la seguridad, y lograr una mejor aceptación social, además de optimizar la protección, son algunos de los retos. Pero para que estas vacunas puedan ser aplicadas masivamente en acuicultura, la necesidad de mejorar el método de administración es crucial. Aunque la inyección se considera por ahora el mejor sistema, por su eficacia, no resulta adecuada ni posible en peces de pequeño tamaño y en los de mas talla requiere tiempo, manejo de peces y agujas, con el consiguiente estrés en la población vacunada. De entre los métodos alternativos que se están probando la vacunación oral sería la forma ideal de administración si resultase posible añadirla al pienso y lograr alta protección.

Las vacunas orales ofrecen muchas ventajas, tales como la facilidad de administración a grandes poblaciones a lo largo del tiempo y permitiendo su uso en peces de pequeña talla, que están en el momento de mayor susceptibilidad a las infecciones por virus. Hasta ahora los experimentos han sido escasos, incluso a escala de laboratorio. El ensayo de vacunas orales frente al IPNV y al IHNV forma parte de los objetivos de esta tesis. Estas vacunas requieren protección para evitar la desintegración a su paso por el sistema digestivo. En nuestros trabajos se han probado como vacunas recubiertas con sales de alginato, mediante administración directa para determinar protección y respuesta inmune en una población homogéneamente vacunada y mediante incorporación al pienso; en el caso del IPNV se han realizado bien mediante recubrimiento del antígeno con la comida o bien mediante una mezcla de ambos en la producción del alimento.

Actualmente, las vacunas descritas en el ámbito de la acuicultura frente a los virus de IPNV e IHNV, se resumen en la tabla 5.

Tabla 5: Vacunas DNA descritas en peces Salmónidos, frente al IHNV e IPNV

Patógeno	Gen, inserto	Hospedador	Vía de administración/ adyuvante	Protección y referencia
IHNV	Gen G, mas gen suicida	Trucha arco iris	IM/Ninguno	Sí (Alonso et al. 2011)
IHNV	Gen G de diferentes genogrupos	Trucha arco iris	IM/Ninguno	Sí, protección cruzada (Penaranda, LaPatra, and Kurath n.d.)
IHNV	Gen G.	Trucha arco iris	Oral/PLGA	No (Adomako et al. 2012)
IPNV	Gen VP2.	Trucha arco iris	Oral/Alginato	Sí (de las Heras et al. 2010)
IPNV	Gen VP2. de la cepa TA	Salmon Atlántico	IM/Ninguno	No (Munangandu et al. 2012)

Puesto que las vacunas DNA frente a virus de peces teleósteos parecen ser por ahora la única alternativa eficaz posible, los estudios sobre nuevos promotores, métodos de administración oral, perfiles de respuesta inmune innata y su estimulación, así como la búsqueda de nuevas citoquinas que puedan ser co-adyuvantes, son cuestiones que aún precisan diversos diseños y desarrollo experimental en el laboratorio.

Algunos de estos aspectos se han abordado como objetivos de esta tesis Doctoral.

Objetivos

El trabajo de Tesis es parte de los proyectos de Investigación que el Laboratorio de Virus del Grupo de Vacunas y Expresión Génica del Centro de Investigaciones Biológicas viene desarrollando estos últimos años.

El objetivo general de este trabajo es el estudio de la respuesta inmune de vacunas DNA frente a IPNV e IHNV administradas por vía oral en trucha arco iris, con el fin de mejorar, por una parte el sistema de administración y por otra la seguridad; en este último caso, para el IHNV, al sustituir el promotor CMV de origen humano por otro de origen piscícola. Se plantearon los siguientes objetivos:

- ✓ 1. Caracterizar la expresión génica inducida en trucha arco iris por una vacuna DNA oral frente al virus de la necrosis pancreática infecciosa.

- ✓ 2. Optimizar métodos de administración de vacuna DNA oral.

- ✓ 3. Determinar la eficacia de la vacuna pcDNA-VP2 oral en la prevención de estados de persistencia del virus de la necrosis pancreática infecciosa.

- ✓ 4. Evaluar la respuesta inmune y protección inducida por una vacuna DNA oral frente al virus de la necrosis hematopoyética infecciosa.

- ✓ 5. Determinar masivamente los perfiles de transcripción de trucha arco iris en respuesta a la infección con el virus de la necrosis hematopoyética infecciosa.

1. Caracterizar la expresión génica inducida en trucha arco iris por una vacuna DNA oral frente al virus de la Necrosis Pancreática Infecciosa.

Herramientas para determinar la expresión transcripcional: Microarray

Los *microarrays* han evolucionado a partir de la técnica de Southern blot desarrollada en 1975. La tecnología de los arrays fue utilizada por primera vez en 1984 para analizar las terminaciones largas repetidas (LTR) de un retrovirus. Posteriormente, en 1987 se utilizaron los arrays (microarrays de expresión) para analizar la expresión de más de 2000 genes de una línea celular humana de fibrosarcomas con y sin tratamiento de interferón (Kulesh et al. 1987). El Microarray miniaturizado fue descrito en 1995 (Schena et al. 1995), y el primer genoma eucariota completo en un microarray fué descrito en 1997, en el análisis de la expresión génica de *S. cerevisia* (Lashkari et al. 1997). Desde las primeras investigaciones en 1980 se han desarrollado mejores tecnologías como la robotización de los arrays, la mejora de las técnicas, y el desarrollo a nivel computacional, que ha automatizado en gran parte su diseño y análisis, además de disminuir sus costes. Los microarrays de expresión son matrices de material biológico (sondas de oligos– cDNA) ordenado sobre un soporte de silicio o vidrio con una alta densidad de integración, permitiendo la cuantificación de miles de biomoléculas simultáneamente. Dependiendo de la naturaleza de las mismas, las matrices se clasifican en *microarrays* de DNA, RNA (por ejemplo, de RNA de interferencia), de proteínas (por ejemplo, de anticuerpos), de glúcidos, de células y de tejidos.

A finales de la década de 1980, se desarrollaron los primeros *microarrays*, que consistían en matrices de DNA inmovilizado (Deering et al. 1991). El DNA puede ser fragmentado al azar, o cDNA, u oligonucleótidos. Entre sus aplicaciones destaca el análisis de la expresión génica diferencial a nivel global, que consiste en la hibridación de dos poblaciones distintas de RNA o cDNA marcados con fluoróforos diferentes, con la matriz de DNA inmovilizado para cuantificar sus niveles de expresión de modo relativo entre dos muestras biológicas determinadas. En el caso de los *microarrays* de oligonucleótidos destinados al estudio de la expresión génica, es necesario conocer la secuencia de los genes del organismo con anterioridad a su fabricación, que se realiza mediante síntesis de la colección de oligonucleótidos *in situ*, a diferencia de los *microarrays* genómicos o de cDNA, los cuales se construyen a partir de genotecas o bancos génicos; los *microarrays* pueden ser totales (completos) o parciales, dependiendo del grado de representación de las secuencias genómicas de las que proceden (Kassahn 2008).

En algunos organismos la primera limitación al utilizar esta técnica consiste en el conocimiento previo de la secuencia del genoma; también los altos niveles de fondo debido a la hibridación. El número limitado del rango de detección debido al fondo y la saturación de las señales, requiere métodos de normalización complicados para comparar los niveles de expresión a través de diferentes experimentos. Además, sólo una parte de la transcripción es analizada y no es posible detectar diferentes isoformas (Schena et al. 1995).

1.1. Estudios de perfiles transcriptómicos de la respuesta inmunitaria en truchas vacunadas.

En trabajos previos se ha observado la inducción de anticuerpos neutralizantes y protección frente a IPNV de vacunas DNA por vía oral recubierta con alginato en trucha arco iris (Ver figura 1). Sin embargo, no se habían descrito los perfiles de transcripción de respuesta inmune en truchas vacunadas. El primer paso consistió en establecer relaciones causales directas entre genes concretos que participan activamente o regulan una respuesta optima de inmunización por vía oral utilizando una vacuna de DNA, para lo cual se utilizaron microarrays de oligonucleótidos de trucha arco iris de 60-mer oligo 8x15K (ID032303) con 6442 genes o 60-mer secuencias de oligos diseñadas por el Dr. Julio Coll (INIA). El diseño de este microarray está depositado en el NCBI en la base de datos de GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=y 31.557) y fabricados por Agilent. El diseño final ID32303 contiene un total de 3106 sondas, las cuales fueron duplicadas y distribuidas al azar en la placa de silicio.

Vacunación por vía oral en peces

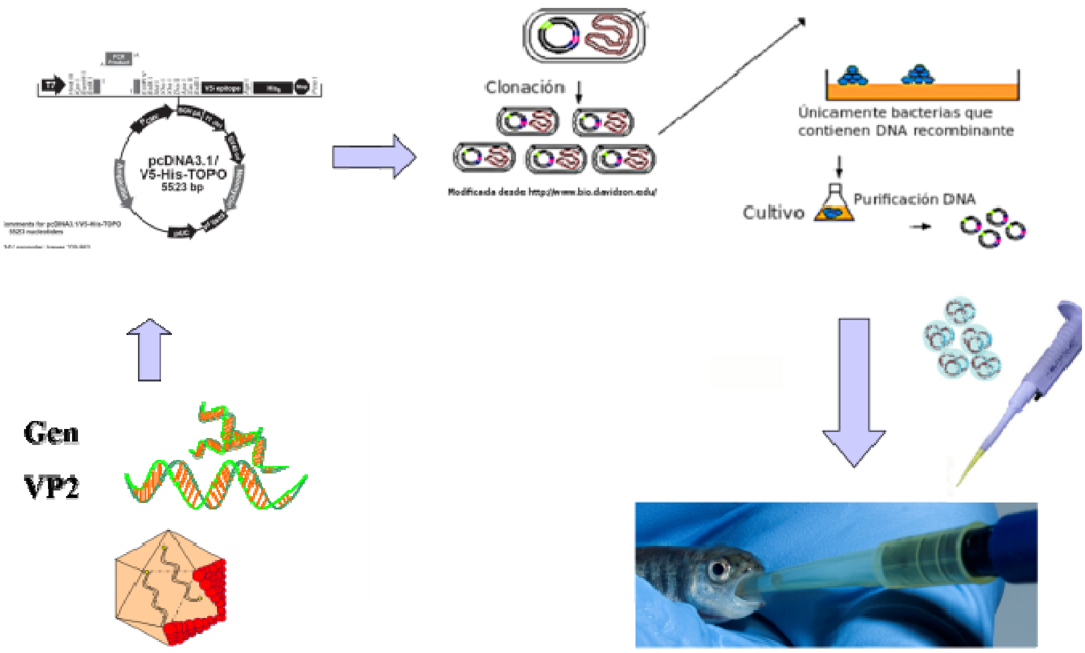

Figura 1: Esquema de la producción de la vacuna de DNA e inmunización de las truchas por vía oral según de las heras (2010) (de las Heras et al. 2010).

Diseño experimental:

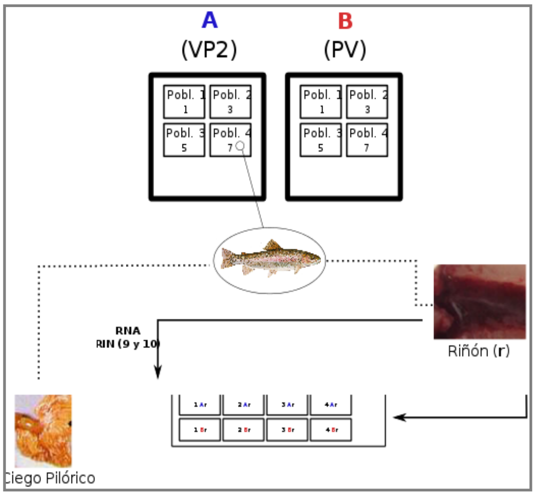

Figura 2: Esquema del experimento llevado a cabo mediante microarrays.

Alevines de trucha arco iris de aprox., 1g, 3,5 cm de longitud. Peces de cuatro poblaciones diferentes. Ver figura 2.

- Grupo de peces n=15 vacunados con 10 µg pcDNA-VP2 por vía oral, “vacunados”.
- Grupo de peces n=15 vacunados con 10 µg pcDNA (plásmido vacío) vía oral “plásmido vacío”.
- Grupo de peces sin tratamiento n=15 “Control”.

Tiempo de muestreo: 7 días post-tratamiento

Muestras: riñón anterior (n=6) y ciego pilórico (n=6)

Métodos: Extracción de RNA total, Análisis de calidad de RNA por sistema de electroforesis automatizada (Experion, BioRAd) Microarray de Agilent de Trucha.

Resumen

Una vez obtenido el RNA de los tejidos, se evaluó su integridad y concentración como se muestra en la figura 3.

Las muestras que presentaron índices de calidad, RQI (RNA quality indicator) entre 9 y 10 (Ver figura 3) se hibridaron, tiñeron y se leyeron utilizando el servicio de la empresa Ningenetics (Madrid, España).

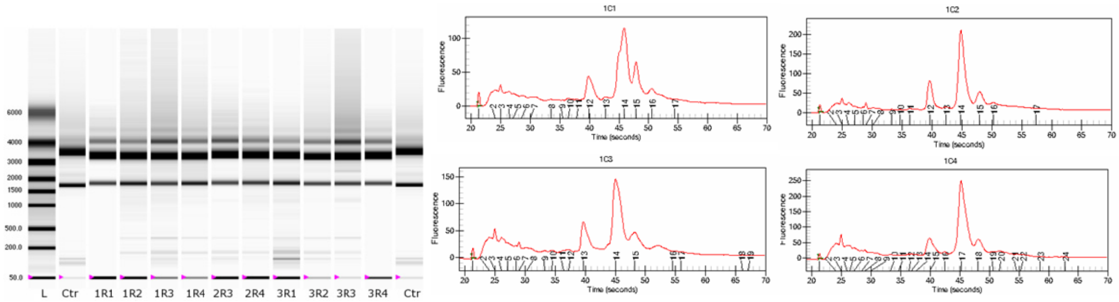

Figura 3: Resultados de electroforesis automáticas (Experion-Biorad) de RNA extraído de riñón anterior.

Tras la hibridación del RNA al microarray; la señal de fluorescencia fue capturada y procesada utilizando un escáner de Agilent (G2565B, Agilent Technologies) y el Software Agilent Feature Extraction Software (v9.5) protocolo GE1-v595.

Según las características predeterminadas por la casa comercial, los datos de los pixeles con valores extremos, al igual que los valores de replicas con valores atípicos fueron desechados (gIsFeatNonUnifOL, gIsBGNonUnifOL, gIsFeatPopnOL, gIsBGPopnOL); la relación entre la señal procesada y el error fue de 2 para eliminar la señal de fondo.

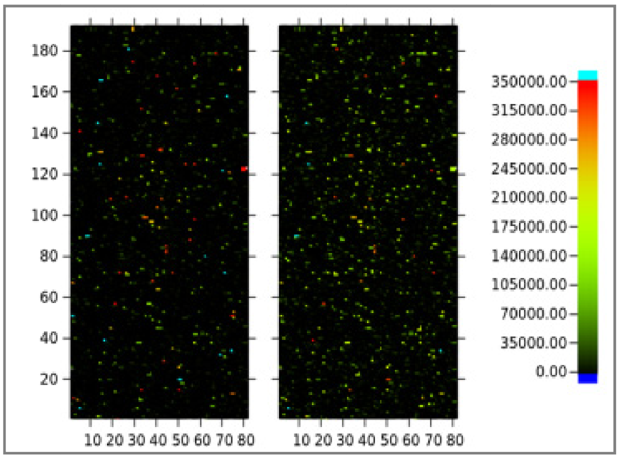

Figura 4: Representación gráfica de la intensidad de la fluorescencia (gProccesedSignal) captada y procesada por el escáner de Agilent. La variación en la intensidad de la señal de los genes expresados se grafica entre 10 a 1000000 unidades fluorescentes relativas.

La normalización de la intensidad de los genes expresados "gProccesedSignal" (Figura 4), fue llevada a cabo de acuerdo a las formulas mostradas en la sección 1.5 de "herramientas desarrolladas (Gene2Path)". Se consideraron genes expresados (sobre-expresados) o inhibidos (sub-expresados) cuando el valor de expresión de dos grupos comparados (valores de pcDNA-VP2 con pcDNA-) era igual o mayor a 4 veces (fold).

Para el análisis estadístico se utilizaron dos criterios de identificación de genes diferencialmente expresados:

- Los ratios de los genes de las truchas inmunizadas con pcDNA-VP2 *versus* (vs) truchas expuestas a pcDNA deben ser mayor a 2, y
- Los genes que rechazaron la hipótesis nula utilizando la prueba t de $p<0,05$.

Los cálculos de expresión génica de cada órgano estudiado se realizaron a partir de valores de cuatro muestras o repeticiones biológicas. El procesamiento de los datos se llevó a cabo utilizando el software Origen Pro vs. 8,5 (Northampton, EE.UU.) y un programa que se ha diseñado en el grupo, mencionado en la sección 1.5 de "herramientas desarrolladas con el fin de comprobar los resultados ("Gene2Path", Ballesteros et al 2015) Nº de propiedad intelectual y acta 1018/2012 del 26 de Julio 2012 por el CSIC en la Notaria de Dña. Isabel Griffo.

Como resultados más relevantes de este trabajo, se encontró un incremento en la expresión de los genes inmunes transcritos en el riñón anterior respecto al observado en el ciego pilórico.

En riñón anterior se observó una sobre-expresión de genes correspondientes a los grupos : 1) Interferones, sus receptores y proteínas inducidas (n=91 genes), 2) Vigs, genes inducidos por VHSV (n=25) 3) genes relacionados con macrófagos (n=125) 4) genes del sistema de complemento (n=176) 5) TLR, receptores toll-like (n=31) 6) TNF factor de necrosis tumoral (n=32) 7) quimioquinas y sus receptores (n=121), 8) interleuquinas y sus receptores (n=119) En el ciego pilórico éstos de grupos de genes se mantuvieron sin variación o incluso disminuida su expresión.

Con estos resultados, se identifican nuevos marcadores de genes tanto en el riñón anterior como en el ciego pilórico, que pudieron utilizarse para el seguimiento y/o mejora de la inmunidad durante la vacunación oral en peces.

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Oral immunization of rainbow trout to infectious pancreatic necrosis virus (Ipnv) induces different immune gene expression profiles in head kidney and pyloric ceca

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ABSTRACT

Induction of neutralizing antibodies and protection by oral vaccination with DNA-alginates of rainbow trout *Oncorhynchus mykiss* against infectious pancreatic necrosis virus (IPNV) was recently reported [1]. Because orally induced immune response transcript gene profiles had not been described yet neither in fish, nor after IPNV vaccination, we studied them in head kidney (an immune response internal organ) and a vaccine entry tissue (pyloric ceca). By using an oligo microarray enriched in immune-related genes validated by RTqPCR, the number of increased transcripts in head kidney was higher than in pyloric ceca while the number of decreased transcripts was higher in pyloric ceca than in head kidney. Confirming previous reports on intramuscular DNA vaccination or viral infection, *mx* genes increased their transcription in head kidney. Other transcript responses such as those corresponding to interferons, their receptors and induced proteins ($n = 91$ genes), VHSV-induced genes ($n = 25$), macrophage-related genes ($n = 125$), complement component genes ($n = 176$), toll-like receptors ($n = 31$), tumor necrosis factors ($n = 32$), chemokines and their receptors ($n = 121$), interleukines and their receptors ($n = 119$), antimicrobial peptides ($n = 59$), and cluster differentiation antigens ($n = 58$) showed a contrasting and often complementary behavior when head kidney and pyloric ceca were compared. For instance, classical complement component transcripts increased in head kidney while only alternative pathway transcripts increased in pyloric ceca, different β-defensins increased in head kidney but remained constant in pyloric ceca. The identification of new gene markers on head kidney/pyloric ceca could be used to follow up and/or to improve immunity during fish oral vaccination.

1. Introduction

Oral delivery is considered the most desirable way to vaccinate both humans and animals [2,3]. Nevertheless, despite many efforts to find suitable fish oral vaccination methods, there are yet few reports describing their successful use. However, recent reports using either pathogen-coding DNA in trout [1] and in Japanese flounder (*Paralichthys olivaceus*) [4] or pathogen recombinant proteins in salmon [5,6], suggest fish oral vaccination might be possible in the future.

Oral vaccination methods are needed because present oil-adjuvanted vaccines delivered by intraperitoneal injection have important side effects on fish welfare [7–9], while the present licensed DNA salmon vaccines in Canada still require fish-to-fish intramuscular injection [10]. Furthermore, small immunocompetent fish to be vaccinated cannot be injected. However, oral vaccines have many difficult-to-obtain requirements such as to be protected from stomach digestion by some antigen-encapsulation method, adhere to fish guts, avoid induction of immune tolerance or induce immune responses in both local epithelial surfaces and internal organs. Because most of those necessary immune responses are not yet known, we have focused this study on trying to clarify those. In order to do that, the recently reported successful oral immunization of rainbow trout *Oncorhynchus mykiss* against infectious pancreatic necrosis virus (IPNV) with a DNA vector coding for the VP2 capsid gene of infectious pancreatic necrosis virus (IPNV), has been used. Alginate microspheres protected the DNA, which was expressed early and late in different organs of the vaccinated trout, induced neutralizing antibodies and protected 80% of the vaccinated trout [1,11].

IPNV is an economically important *Birnaviridae* which causes severe acute lethal infections in young salmonid fish, remaining one of the most widespread causing-disease virus in aquaculture

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[12,13]. The IPNV genome consists of two double-stranded RNA segments (A and B) that encode VP1 (a RNA-dependent RNA polymerase), VP2 (the major antigenic capsid protein and type-specific antigen with a great antigenic diversity), VP3 (an internal capsid protein and group-specific antigen), NS and VP5 (non structural proteins). The accepted serotyping of IPNV includes two serogroups, the first containing nine serotypes from fish (A_1–A_9) and the second containing a single serotype (B_1) [14]. Because following a disease outbreak, surviving fish may become asymptomatic carriers for life; broodstock carriage is considered an important source of IPNV for lethal infection of hatchery-reared fry. The development of an effective vaccine is a necessity to secure the future of salmonid (salmon and trout) farms.

To study the transcriptional profile of rainbow trout organs after successful oral immunization, we used a newly designed 60-mer oligo microarray enriched in immune-related genes. We studied not only one of the most important fish internal organs involved in fish responses to infection (head kidney) but also one of the entry sites of the vaccine: the pyloric ceca (mucosal immunity). The head kidney was selected as the target internal organ because other reports have demonstrated the presence of DNA at different times after oral vaccination in Atlantic salmon [15] or brown and rainbow trout [1,11]. On the other hand, pyloric ceca could offer a novel approach to study the immunity response at the gut mucosa, which might be especially important for oral vaccination. Results confirmed the induction of *mx1* transcripts in head kidney reported earlier for this and for other immunization methods. More immune-related transcripts increased in head kidney than in pyloric ceca after oral immunization. The study contributed to clarify the immune response to oral vaccination and allowed the identification of novel genes which can be used as markers to improve oral vaccination in fish.

2. Materials and methods

2.1. Preparation of the IPNV-VP2 DNA vector

The plasmid DNA vector (pcDNA-VP2) was prepared as described previously [1]. Briefly, the IPNV-VP2 gene was cloned into the pcDNA3.1/V5/His-TOPO plasmid (pcDNA) (Invitrogen) under the control of the immediate-early CMV promoter and amplified in *Escherichia coli* TOP10. The plasmid DNA was isolated with the Endofree Plasmid Maxi purification Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration was measured in a spectrophotometer before it was aliquoted and conserved at −20 °C. The pcDNA was used as control plasmid.

2.2. Preparation of microspheres and formulation of the oral vaccine

The procedure for the preparation of the microspheres was described previously [1]. Briefly, 2.5 mL of 3% (w/v) of sodium alginate were mixed with 1.5 mL of 1 mg/ml of pcDNA-VP2 and the mixture stirred at 500 rpm for 10 min. This solution was then added to an Erlenmeyer flask containing 100 mL of paraffin oil and 0.5 mL Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared by drop-by-drop adding 2.5 ml of 0.15 M $CaCl_2$ to the emulsion and stirring for 2 h at 900 rpm. Microspheres were then collected by centrifugation at 1000*g* for 10 min, and were washed twice with 70% ethanol, lyophilized and stored at 4 °C.

2.3. Oral vaccination of rainbow trout

Rainbow trout (*O. mykiss*) of a mean weight of 1 g (mean size of 3.5 cm) were purchased from a spring water local farm with no history of viral disease. Two pools of 5 fish were tested by standard methods to confirm the absence of IPNV or any other salmonid virus by isolation using BF cells [16]. The trout were acclimatized for 2 weeks and kept under a 12/12 h light/dark regime at 15 °C in 350 L closed re-circulating water tanks (Living Stream, Frigid Units Inc., Ohio) at the "Centro de Investigaciones Biológicas" (CSIC, Madrid, Spain). Groups of 20 trout were maintained in separate 45 L tanks supplied with non-chlorinated water using exterior carbon filters (Eheim) and additional aeration. The trout were fed daily with a diet of commercial pellets. Trout were obtained from a unique farm but from 4 different trout populations, one population per group. Each of the 4 groups was divided into 2 subgroups of 6 trout each. First subgroup was orally vaccinated with 10 μl of suspension of the vaccine microspheres each containing 10 μg of pcDNA-VP2 diluted in 10 μl of PBS, while second subgroup received similar amounts of microspheres with pcDNA. Vaccination was performed with an automatic pipette with a 20 μl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the esophagus. The water-quality parameters were maintained at optimum levels and the conditions in all tanks were equal.

The trout were anaesthetized by immersing in 50 mg/mL tricaine-ethanesulfonate (MS-222, Sigma, Madrid, Spain) buffered in PBS prior to handling. After decapitation, the head kidney and pyloric ceca were harvested from each trout. The organs were immediately immersed in RNAlater (Ambion, Austin, USA) and kept at 4 °C overnight before being frozen at −70 °C until processed. Experimental protocols were performed with the approval of the CSIC ethical committee.

2.4. RNA extraction of trout head kidney and pyloric ceca

RNA was extracted from each individual trout head kidney and pyloric ceca after sonication (1 min × 3 times at 40 W in ice) in the RTL buffer and by using the RNAeasyPlus kit (Qiagen, Hilden, Germany). RNA concentrations were estimated by nanodrop and the presence of 18 and 28 S bands confirmed by denaturing RNA agar electrophoresis (Sigma, Che.Co, MS, USA). Stringent RNA quality control was performed prior to hybridization. For each group, 4 of the best quality RNA per group were pooled and further analyzed.

2.5. Design of oligo microarrays enriched in rainbow trout immune-related genes

To design the immune-related gene enriched microarray used in these experiments, rainbow trout sequences were selected from both GenBank and Agilent's EST-derived oligo microarray (ID16271) [17]. The immune-related genes were retrieved by using the following keywords: interferon, chemokine, interleukin, cytokine, defensin, macrophage, lymphocyte, antimicrobial, neutrophil, leukocyte, cytotoxic, natural killer, antiviral, antibacterial, LPS, Vig, antigen, histocompatibility, phagocyte, viral, Mx, complement, immunoglobulin, hepcidin, IgG, IgM, Toll, T cell, B cell, dendritic, presenting, TNF, perforin, MHC, NK, transcription, chaperone, stress, Hsp, Hsp70, Hsp90, tlr, flagellin, keratinocyte, cathepsin, NOD, IRF, IKK, JBK1, TRAM, TAK, TAB, JNK, P38, AP-1, TIRAP, IgT, IgH and high mobility. To simplify the analysis of results, the probes were classified according to 16 groups: AM, antimicrobial peptides; C, complement components; CD, cluster differentiation antigens; CK, chemokines; HSP, heat shock proteins; IFN, interferons; IG, immunoglobulins; IL, interleukins; MA, macrophage; MHC, major histocompatibility; MX, interferon-inducible proteins mx; TCR, T cell receptors; TLR, toll-like receptors; TNF, tumor necrosis factor; TR, transcription factors; and VIG, VHSV-induced genes. The resulting list of retrieved gene accession numbers and/or genes and sequences was formatted in excel, duplicates eliminated and the final list submitted to Agilent's microarray design tool (eArray application https://earray.chem.agilent.com/earray/search.do?search1/4arrayDesign). The final 8x15K Agilent design called minitrout12.8 (ID032303) contains 6442 unique 60-mer oligo sequences each in duplicate randomly arranged in the microarray. The microarrays were synthesized *in situ* by using Agilent's non-contact inkjet technology. The final design is available for review free of charge for registered users of the Agilent eArray application (Table 1). A complete list of gene IDs and gene names is included in Gene Expression Omnibus (GEO) platform submission number GPL14155. The microarrays are available from Agilent Technologies.

2.6. Quantitation of immune-related rainbow trout transcripts by microarray hybridization

Two rainbow trout 60-mer oligo 8x15K format custom microarrays (ID032303) containing 6442 60-mer oligo sequences was obtained from Agilent. RNA was kept frozen at -80 °C until all the experiments were hybridized and processed simultaneously. Labeling of 2 μg of RNA ($\sim$50 μg/ml) and hybridization to the microarrays were performed by the NimGenetics company (Madrid, Spain) complying with the Minimum Information About a Microarray Experiment (MIAME) standards. Briefly, high quality RNA were labeled with Cy3 (Amersham Pharmacia) by using SuperScript III reverse transcriptase (InVitroGen) and oligo(dT) primer, and the resulting cDNA was purified with Microcon YM30 (Millipore). The slides were pre-treated with 1% BSA, fraction V, 5 $\times$ SSC, 0.1% SDS (30 min at 50 °C) and washed with 2 $\times$ SSC (3 min) and 0.2 $\times$ SSC (3 min) and hybridized overnight in cocktail buffer containing 1.3 $\times$ Denhardt's, 3 $\times$ SSC 0.3% SDS, 2.1 μg/μl polyadenylate and 1 μg/μl yeast tRNA. The fluorescence signal was captured, processed and segmented using an Agilent scanner (G2565B, AgilentTechnologies) by using the Agilent Feature ExtractionSoftware (v9.5) with the protocol GE1-v5_95, extended dynamic range and preprocessing by the Agilent feature extraction. Normalization within each microarray was carried out by using the sum of all the fluorescences and results deposited in the GEO bank (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31591 and 31557). The gProccesedSignal was chosen for statistical analysis. Data was first filtered by non-uniform pixel distributed outliers and other replicate outliers (glsFeatNonUnifOL, glsBGNonUnifOL, glsFeatPopnOL and glsBGPopnOL) according to the default Agilent feature extraction criteria; ratio between processed signal and its error <2; differentiation from background signal; linear relationship between concentration and/or intensity below limits according to Spike-In information. Then biological replicate outliers were defined as values outside the mean $\pm$ standard deviation. They were masked from calculations by using a homemade program in Origin vs8.5 (Northampton, USA). After removing outliers, control values from trout immunized with control pcDNA were averaged and fold calculated by the following formula, values from trout immunized with pcDNA-VP2/mean values from trout immunized with pcDNA, $n = 4$. Outliers in the calculated folds were again eliminated before calculating the final mean and standard deviations. The Student's t one tail statistic associated p was computed from outlier-free values obtained from trout immunized with pcDNA-VP2 and trout immunized with control pcDNA for each immune-related gene. A double simultaneous criterion was used to identify differentially expressed genes: (i) genes with ratios IPNV-infected/non-infected >2 and (ii) genes which deviated from the null hypothesis using the t-test at $p < 0.05$. Calculations were made from four biological replicates of pcDNA-VP2 and pcDNA each and by two independent researchers using Origin pro vs 8.5 (Northampton, USA) and their results confronted until all discrepancies were solved.

2.7. Quantitative estimation of transcripts for selected immune-related genes by RTqPCR

RNA was extracted as described above. For RT quantitative real time PCR (RTqPCR), we primed 5 μg of total RNA with 25 pmol/μl Oligo-d(T) and used the Super Script™ II kit (Invitrogen, USA) for reverse transcription. qPCRs were then performed by using SYBR green, in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The qPCR amplifications were carried out in 96-well plates by mixing 5 μL of 20-fold diluted cDNA, 12.5 μL of 2$\times$ concentrated iQ SYBR® Green Supermix (Bio-Rad), 0.3 μM forward primer and 0.3 μM of reverse primer in a 25 μl reaction volume for each sample. The thermal profile was 10 min at 95 °C, followed by 40 amplification cycles of 10 s at 95 °C and 1 min at 60 °C and a dissociation cycle (1 min at 95 °C and 1 min at 60 °C). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. The elongation factor 1α (*EF-1α*) gene was used as house keeping gene in each RNA sample in order to normalize the results to eliminate variation in mRNA/cDNA quantity and quality. No amplification product was observed in controls containing no RNA samples. All qPCR reactions were performed in triplicate and the results were expressed as mean $\pm$ standard deviation. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) and the

Table 1
Number of *Oncorhynchus mykiss* immune-related gene sequences in GenBank (list of probes selected by the eArray program of Agilent) and in the ID16271 microarray [17].

Short name	Groups of genes	GenBank sequences	Agilent eArray list	Previous ID16271	Final design ID32303
AM	Antimicrobials and apoptosis	44	28	30	58
C	Complement components	69	46	130	176
CD	Cluster differentiation	50	37	21	58
CK	Chemokines and receptors	92	80	41	121
HSP	Heat shock proteins	107	88	159	247
IFN	Interferons and receptors	56	46	45	91
IG	Immunoglobulins and receptors	901	814	100	914
IL	Interleukins and receptors	94	75	44	119
MA	Macrophages	41	30	95	125
MHC	Major histocompatibility complex	272	252	68	320
MX	Interferon-inducible Mx proteins	6	6	1	7
TCR	T cell receptors	119	114	6	120
TLR	Toll-like receptors	26	16	15	31
TNF	Tumor necrosis factor	39	25	7	32
TR	Transcription factors	289	210	461	671
VIG	VHSV-induced genes	37	25	1	26
0.5					
Total		2242	1892	1214	3106

expression of target genes was calculated as relative folds of the expression of pcDNA controls according to the $2^{-\Delta\Delta CT}$ method [18].

3. Results

3.1. Design of oligo microarrays enriched in rainbow trout O. mykiss immune-related genes

The GenBank immune-related genes retrieved by using selected keywords were cured by eliminating duplicates and non-appropriated sequences to a total of 2242 sequence entries (Table 1). Those were submitted to the Agilent eArray design application to recover 1892 (84.3%) unique 60-mer oligo probes. The same keywords were also used to extract 1214 immune-related probes from the previously published EST-derived oligo microarray (ID16271) [17]. The final ID32303 design contained a total of 3106 probes, which were randomly distributed in the microarray in duplicates. Table 1 shows the number of probes classified in groups of genes obtained in the intermediate steps to get to the final version of the microarray. The probes retrieved from the GenBank resulted in an overall 2.5-fold enrichment in immune-related genes of the previous ID16271 trout microarray with only 2.4 % of repeated probes between both designs. There were a higher number of probes in the IG and TCR gene groups due to the abundant number of sequences from the variable regions which were in the GenBank. The CD, CK, IL, MX, TNF and VIG groups of genes were the groups where the enrichment was higher (Table 1).

3.2. Genes identified by hybridization to rainbow trout O. mykiss microarrays

Fig. 1 shows a comparison of the distribution of the mean of fluorescence signals reflecting transcript expression levels obtained from trout immunized with pcDNA-VP2 and pcDNA and distributed among the different gene groups in head kidney and pyloric ceca. The signal intensities ranged from 10 to 1,000,000 fluorescent relative units. The distribution of signal intensities shows that more fluorescences from head kidney were >1-fold (above the white line in the figure), than for pyloric ceca.

Fig. 2 shows the distribution of the differential expression with folds >2 (pcDNA-VP2/pcDNA) of transcripts classified by gene groups (Table 1) in both head kidney and pyloric ceca. In the head kidney, the percentage of transcripts with increased expressions was higher than the percentage of genes with decreased expressions while in pyloric ceca the percentage of transcripts with increased expressions was lower than the percentage of genes with decreased expressions except for the IFN and IL groups.

Expression levels of genes from head kidney or pyloric ceca were first selected and tabulated under highly restrictive conditions such as folds >4 and $p < 0.05$. Then, those folds of the corresponding genes of pyloric ceca or kidney, respectively, were also tabulated for comparison. The increased (+) or decreased (−) differential expression of transcripts from genes under those highly restrictive conditions is shown in the Supplementary data. Again, most of the increasing differential expression genes were found in head kidney when compared to pyloric ceca (68.8% compared to 7.8%, $n = 74$) and most of the decreasing differential expression genes were found in pyloric ceca when compared to head kidney (24.6% compared to 3.9%, $n = 74$), confirming the general tendency of the transcriptional data commented above. The genes showing more than 9-fold differential expression in head kidney were the antimicrobial peptide *leap2a*, chemokine *cxcd1*, *procathepsin B*, *il11*, transcription factor *dermo1* and the acute phase protein *pentraxin* (increased) and the chemokine *scya113* and transcription factors *jnk2* (decreased), while in pyloric ceca were the *il10* and transcription factors *lectin* and *zn503* (decreased).

A more detailed analysis of the differential expression of transcripts was undertaken group by group. Thus, Fig. 3 (interferons, their receptors and induced proteins, $n = 91$ genes), Fig. 4 (*mx*, $n = 6$ and VHSV-induced genes, $n = 25$), Fig. 5 (macrophage-related genes, $n = 125$ and complement component genes, $n = 176$), Fig. 6 (toll-like receptors, $n = 31$ and tumor necrosis factors, $n = 32$), Fig. 7 (chemokines and their receptors, $n = 121$ and interleukines and their receptors, $n = 119$), and Fig. 8 (antimicrobial peptides, $n = 59$ and cluster differentiation antigens, $n = 58$) show the main results of those groups of immune-related genes.

Head kidney 9 *ifn* gamma-inducible transcripts corresponding to 7 different genes (*i-p30*, *i-gig2*, *i-hep*, *i-p35*, *i-p58*, *i-p2*, and *i-gtp*), *ifn* gamma receptor (*ifn gr1*) and *type 1 ifn* were increased while *type 1 ifn* a, 3 and 4 were decreased. In contrast, pyloric ceca 2 *ifn* gamma-inducible and 4 *ifn* gamma transcripts (corresponding to genes *ifng1* and *ifng2*) were increased while an *ifn*-inducible protein 30 (*i-p30*) and interferon regulatory factor (*irf1*) transcripts were decreased (Fig. 3).

Head kidney, *mx1*, *2* and *3* transcripts were increased while in pyloric ceca only *mx3* was increased to a similar level than in head kidney (Fig. 4 up). No *mx*-related transcripts were decreased in any of the organs tested.

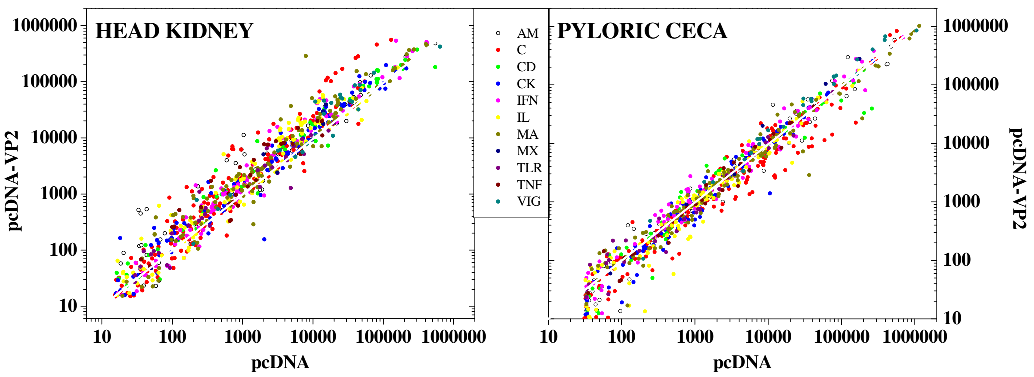

Fig. 1. Comparison of fluorescence intensities in genes from head kidney or pyloric ceca obtained by hybridization to microarrays of transcripts from pcDNA-VP2- and pcDNA-orally vaccinated trout. The trout ID32303 microarray described in Table 1 was used to estimate differential transcript levels in head kidney or pyloric ceca in orally vaccinated rainbow trout. Assay conditions and analysis were as described in Section 2 and Supplementary data. The figure shows the range of mean fluorescences obtained from 4 different pools of trout ($n = 6$ per pool) classified in the gene groups described in Table 1, except those corresponding to the most abundant IG, MHC, TCR, HSP and TR groups which were omitted for clarity. A white straight line has been drawn to show the fold =1.

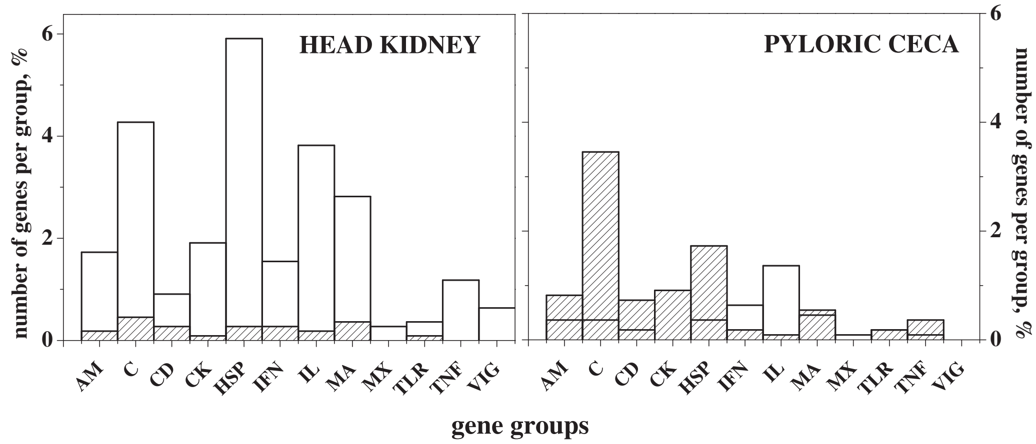

Fig. 2. Distribution of percentages of grouped genes showing differential expression with folds >2 in head kidney and pyloric ceca after oral vaccination. Assay conditions and analysis were as described in Section 2. For each of the gene groups, the percentages of genes showing differential expression were calculated by the formula, 100 × number of genes in the group with folds >2/total number of genes (Table 1). Up: genes showing increased folds (open bars). Down: genes showing decreasing folds (hatched bars). The IG, MHC and TCR groups have been omitted for clarity.

Head kidney *Vig* transcripts *b191*, *b88*, *4*, *5* and *1* were increased, however, neither head kidney nor pyloric ceca *vig* genes decreased (Fig. 4 down).

Among the 125 gene probes classified as macrophage-related, transcripts from the head kidney pentraxin (an acute phase response protein similar to C-reactive protein) was increased >30-fold. In addition, 3 probes of *cd11* (a dendritic cell marker), 3 of macrophage stimulating factor (*csf*) probes, and one each of stabilin (scavenger receptor), cadherin (an adhesive molecule involved in calcium-dependent cell to cell adhesion) and leucocyte immune-type receptor detected increased transcripts. In pyloric ceca, only the 3 *cd11* transcripts were also increased while *stabilin* and *lect2* were decreased (Fig. 5 up).

Head kidney transcripts corresponding to the classic complement pathway (i.e.: *c1r/cls*, *c3*, *c5*, *bf2*) and to the downstream membrane attack complex (*c6*, *c7*, *c8*, *c9*) were increased including one complement receptor (*cr*). Both precursor and mature *properdin* (main actor in the alternative pathway) transcripts and factor *H* were also increased. In the pyloric ceca, in contrast, only the *properdin* and *perforin* transcripts remained increased while those corresponding to the factor *H* gene together with the *c3*, *bf2*, *cfh*, and *cr* were decreased (Fig. 5 down).

In both head kidney and pyloric ceca, differential expression changes in transcripts of pattern recognition toll-like receptor *tlr* members were detected only in a few gene transcripts and with small increments (Fig. 6 up). Thus, in head kidney, *tlr2* (recognizing bacterial lipoproteins and glycolipids), *tlr5m* (located in the membrane of dendritic cells recognizing bacterial flagellins) and an unknown *tlr* and *tlr5m* and *tlr9* (recognizing bacterial CpG DNA) in pyloric ceca, were only slightly increased. Soluble *tlr5* and *tlr8* in pyloric ceca were decreased (Fig. 6 up).

Among the inducers of systemic inflammation of the tumor necrosis factor *tnf* family, main changes in head kidney transcripts with <3-fold increasing changes included 3 probes of *tnf10* (apoptosis inducer), 2 of *tnf11* (dendritic survival factor), *tnf14* (stimulator of T cells), *tnf c* and *tnf a* (most important inducers of systemic inflammation) and *tnfr*. In contrast, in pyloric ceca, only *tnf13* (involved in B-cell development) was increased and *tnf a*, an unknown *trf* and *tnfr* were decreased (Fig. 6 down).

Chemo attractant cytokines (chemokines, *ck*) and their receptors (number of probes tested $n = 121$) showing differential gene expression after VP2 oral vaccination, could be grouped in: *scya* (small inducible cytokine A family-like genes), C, CXC and *socs* (suppressor of cytokines signaling) (Fig. 7 up). In the head kidney, transcripts corresponding to genes similar to *scya109* were increased while those of *scya113* were decreased. In the head kidney, CC chemokines such as *ck3, ck5*, *ck9*, *ck10* and *ck12* were also increased while in pyloric ceca, *ck2* and *ck11* were decreased. In

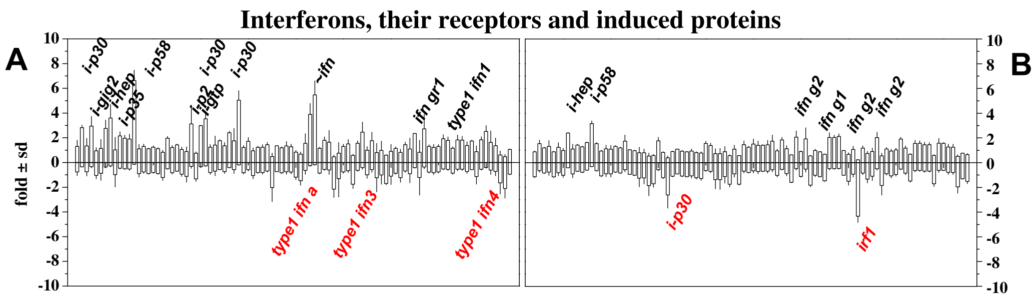

Fig. 3. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of interferons, their receptors and induced proteins. Trout were vaccinated with 10 µg of pcDNA-VP2 or pcDNA ($n = 4$). RNA was extracted from the head kidney and pyloric ceca 7 days after vaccination, labeled with Cy3 and hybridized to the microarrays. After normalization, the mean and standard deviations were represented. Short names of the genes with differential expression folds >2 are to the right of the corresponding bars. Fold, expression level in pcDNA-VP2-vaccinated samples/expression level in pcDNA-vaccinated samples. +, increased folds in black; and −, decreased folds in red. *i*, induced; *r*, receptor; *ifn*, interferon; *g*, gamma; and *p*, protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

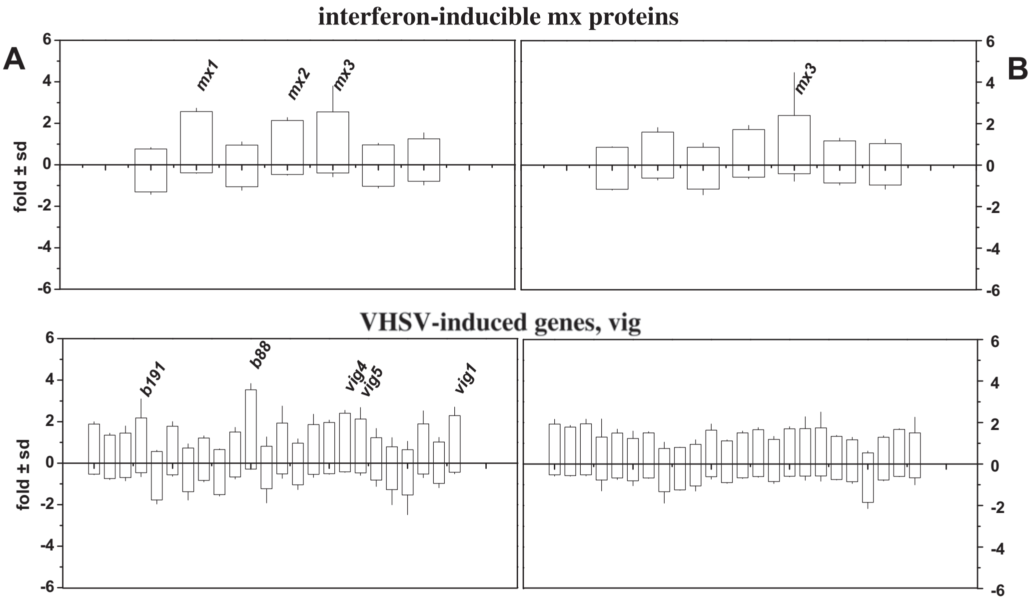

Fig. 4. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of mx (up) and VHSV-induced genes (down). Details as described in the legend of Fig. 3. *vig*, VHSV-induced genes.

the head kidney, the CXC chemokines *cxcd1*, *cxc*, *cxc*r and *il8/il8r* (a CXC chemokine involved in neutrophil chemotaxis, whose results are shown in the interleukines gene group, Fig. 7 down) were also increased while in pyloric ceca, *cxcd1* and *ccr5/cxcr3a* receptors were decreased. Only in head kidney the *socs3* and *socs5* were increased, most probably to control excessive expression levels of other cytokines. In contrast, in pyloric ceca all the differential expression changes detected in chemokine transcripts were decreased. Thus, not only the *cxcd1* (upregulated in head kidney) but also some chemokine receptors (*ccr5, cxcr3* and *r duffy*), *ikhlaii* (a downregulator of *hlaii*), *ck2*, *ck11* and *iNOS* (signaling molecule participating in the oxidative burst of macrophages, among other functions) were decreased.

In the head kidney, of the 119 interleukin and receptors tested, *il10* (inhibits cytokines such as *ifng*, *il2*, *il3*, *tnf* and *csf*), *il12β* (maintains T helper 1 cellular responses to intracellular pathogens), *il17* (pro-inflammatory cytokine produced by activated memory T cells) and *il20* (regulates proliferation and differentiation of keratinocytes) transcripts were increased. In contrast, in pyloric ceca most of the detected transcripts with differential expression levels were decreased (Fig. 7 down). Also increased in head kidney were the *il8* and its receptor (chemokine discussed above), *il11* (inhibitor of apoptosis) and *il13* (pleitropic cytokine involved in the regulation of *ifng*) and the receptors corresponding to *il8*, *il13*, *il20* and *il4* (*il4* is an interleukin produced by *cd4+* cells for B-cell responses). In the pyloric ceca, 4 probes of *il1β* (a lymphocyte mitogen produced by

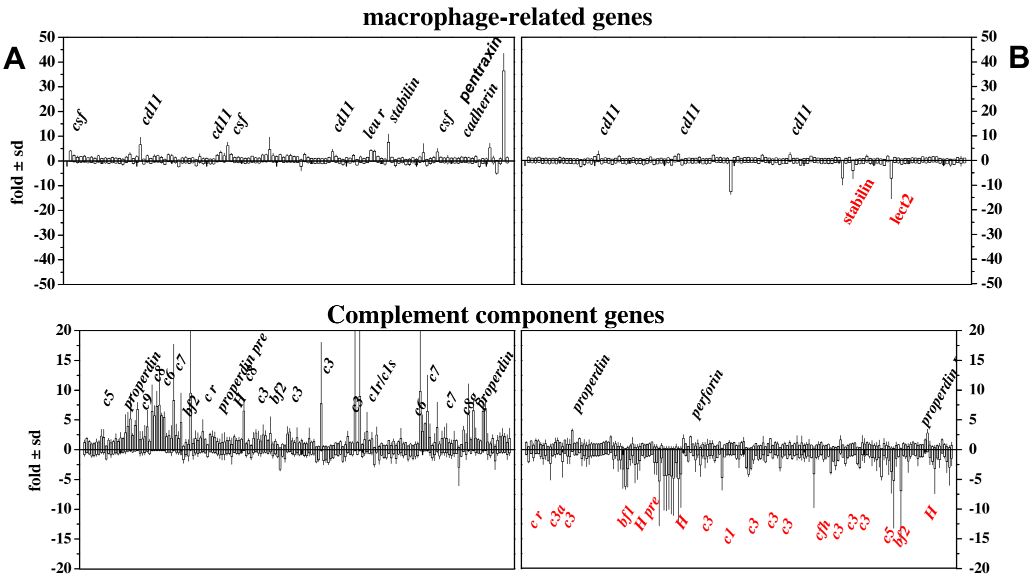

Fig. 5. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of macrophage-related genes (up) and complement component genes (down). Details as described in the legend of Fig. 3. *cd*, cluster differentiation antigens. *r*, Receptor; *c*, complement component; and *H*, factor H.

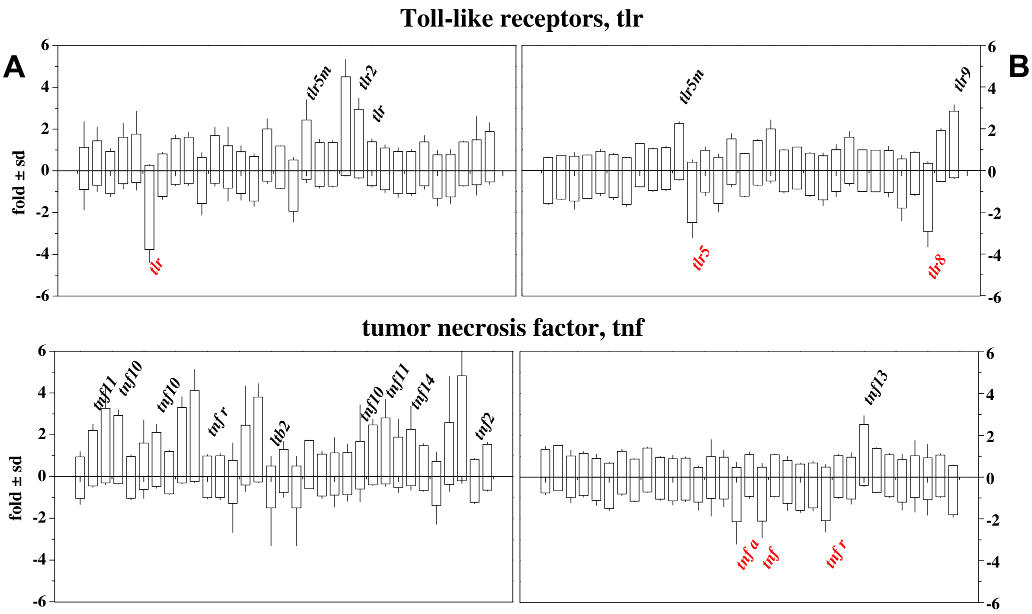

Fig. 6. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of toll-like receptors (up) and tumor necrosis factors (down). Details as described in the legend of Fig. 3. *tlr*, toll-like; *r*, receptor; *m*, membrane; *tnf*, tumor necrosis factor; and *a*, alpha.

macrophages and an important mediator of inflammatory responses), *il6* (B-cell stimulating factor important in the differentiation of B cells into secretory cells) and *il22* (increasing the acute phase proteins) were also decreased.

Fig. 8 (up) shows that in the transcripts grouped in the antimicrobial peptide genes, all the folds were increased much more in the head kidney than in the pyloric ceca. Thus, in the head kidney, transcripts incremented included *leap2a* (2 probes) and *βdef1* (5 probes), *βdef2* and *βdef4* (2 probes), while in pyloric ceca only the *leap2a* and *βdef4* were slightly incremented.

In the head kidney, the *cd* transcripts present in B, T and macrophage cells were increased (Fig. 8 down). Thus, *cd79* (protein associated with membrane-bound immunoglobulin in B-cells) and *cd80/86m* (primordial molecule existing in membrane-bound B-cells), transcripts corresponding to *cd4* (a typical marker of T helper cells) (4 probes), *cd36* and *cd163* precursor (scavenger receptors of activated monocytes and macrophages) and *cd166* and *cd97* (activated leukocyte cell adhesion molecules) were increased. In head kidney, *cd209* (c-type lectin marker of dendritic cells) and the secreted form of *cd80/86* were decreased. In pyloric ceca, only the

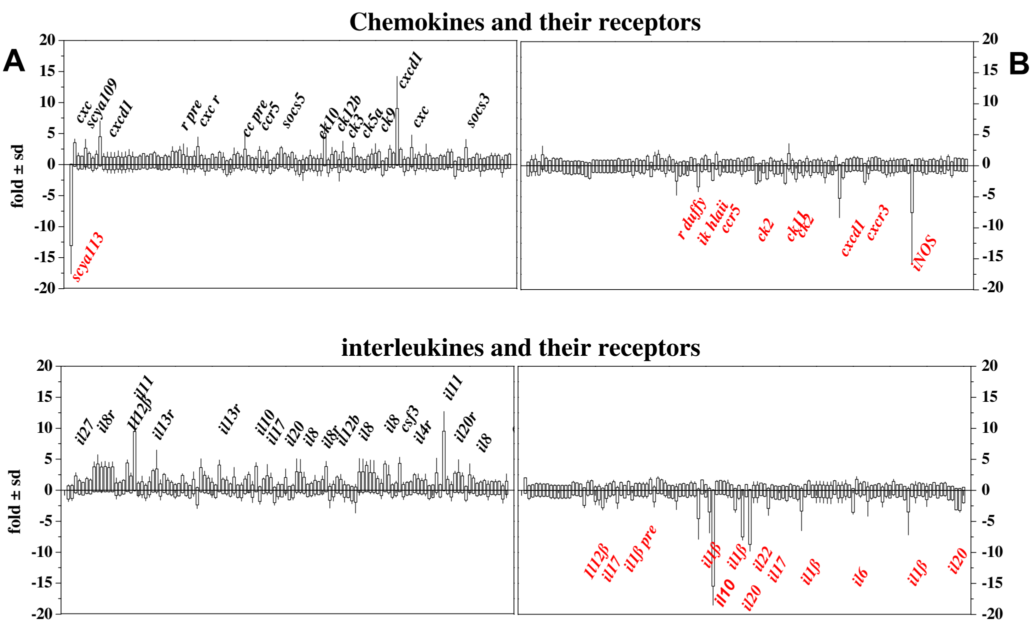

Fig. 7. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of chemokines and their receptors (up) and interleukines and their receptors (down). Details as described in the legend of Fig. 3. *ck*, chemokine; *scya*, small inducible cytokine A; *socs*, suppressor of cytokine signaling; *pre*, precursor; *r*, receptor; and *il*, interleukin.

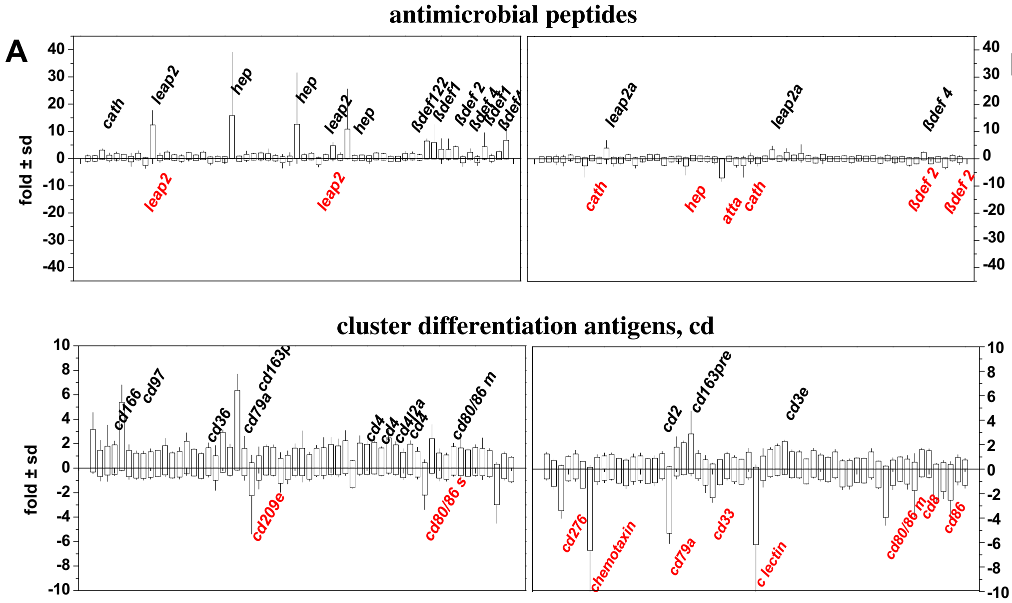

Fig. 8. Hybridization of head kidney (A) and pyloric ceca (B) transcripts to immune-gene enriched oligo microarray. Differential transcript expression of antimicrobial peptides (up) and cluster differentiation antigens (down). Details as described in the legend of Fig. 3. *cath*, cathepsin; *leap*, liver-expressed antimicrobial peptide; *def*, defensin; *cd*, cluster differentiation antigen; and *pre*, precursor.

cd163 precursor was increased together with *cd2* (thymocytes) and *cd3* (signaling component of the T-cell receptor complex) while, chemotaxin (a chemoattractant of leukocytes), *cd8* (marker of cytotoxic lymphocytes), *cd79*, *cd33* (marker of immature myeloid cells), *c-lectin* (cell adhesion and immune response to pathogens), *cd276* (costimulatory molecule of *cd80/86*) and the membrane-bound form of cd80/86 were decreased.

In the head kidney, increased differential transcripts were detected with 3 probes of *hsp70* (folding and unfolding), 3 of *hsp40* (a cofactor of hsp70), 2 of *hsp90* (maintenance of transcription factors) and 3 of prefoldin (*pfd*) (implicated in the correct folding of nascent proteins). Furthermore, different cathepsin (*cath*) proteases were also increased (12 probes). In pyloric ceca, on the contrary, *hsp70* (2 probes), *hsp40* (1 probe) and *hsp90* (2 probes) and *cath* (6 probes), were all decreased while *pfd*s were maintained (Supplementary data and results not shown).

There were no detectable transcript changes in the secreted mu chain of *igm*, nor in the *igt*, nor in the *tcr* in any of the organs studied 7 days after vaccination, except for a decreased level of *tcrb* in pyloric ceca (not shown).

In the head kidney there were transcript increases as detected by multiple probes corresponding to the MHC class I and II precursors, MHC class Ia and Ib (UA, ZE, UBA, UEA and UAA), MHC class II antigen β (DAB, DBB and DAA) and β2 microglobulin. In the pyloric ceca most of those remained unchanged, except MHC class II antigen β which was down-regulated (4 probes).

In the head kidney there were many transcription factors whose transcripts were increased. Thus, general transcription was activated as shown by the increasing transcription levels of genes corresponding to transcription initiation (*tfiid, tfiia* and *iifa*) and elongation (*siiip15*, *siii*, and *spt4*) factors (as detected by several different probes for most of them), several transcription-related-like factors (i.e.: *iib*, *iih*, *lipl21*), CCAAT enhancer binding protein β*c/ebpb* (6 probes) and RNA polymerase-related molecules such as RNA polymerase I cofactor (*rrn3*) and termination factor (*ttf1*) and RNA polymerase III polypeptide (*rpc11*). In pyloric ceca although a few general transcription factors decreased (i.e.: *iif* and *tfiih*), most of them remained unchanged. Among the *sox* family (a family of transcription factors containing an hmg DNA-binding domain), the *sox21* (counteracts *sox1*, *2*, *3*), the *sox30* and the *sox*-related *sry* were increased. In pyloric ceca most of the *sox* transcripts were decreased except the *sox21.* Other transcription factors increased in head kidney were *ap1* (stimulated by different cytokines and viral infections), which remained increased in pyloric ceca, and the *g0s2*, also called the lymphocyte G0/G1 switch protein (whose expression is required to commit lymphocytes to pass from the G0 to enter the G1 phase of the cell cycle to start division). Strikingly the *jnk2* N-terminal protein kinase was >10-fold decreased in head kidney while it was 8-fold increased in pyloric ceca (Supplementary data). The *jnk2* has been linked to *tnf* levels and apoptosis [19,20], but it is not known why it is expressed so differently in the studied organs.

3.3. Selected gene expression by RTqPCR

To validate the results of the microarray analysis, some of the genes commented above were selected for RTqPCR analysis. Genes previously described as induced in IPNV infected fish were also included. The selected genes were: *ifn1* [21], *ifng* [22], *mx1* [23] and *3* [24–26], *stat1* [27], *mhc1* [28], *tnf2* [29], *il8* [30], *1l10* [31], *1l11* [32], *1l12* [33], *βdef1* [34], *igm* [35], and *igt* [36]. Table 2 represents the selected genes, their accession numbers and the corresponding primer sequences used for the RTqPCR analysis. Fig. 9 shows the comparison between the folds of transcripts obtained by microarray and by RTqPCR corresponding to either head kidney (A) or pyloric ceca (B). The folds were similar for the microarray and the RTqPCR for the majority of each of the gene data, except one data >3.5-fold in head kidney and two data >5-fold in pyloric ceca. RTqPCR results show that in head kidney *stat1a*, *il11*, *il12* and *il10* were more increased than in pyloric ceca while *ifn1*, *ifng*, *mx3*, *igm*, and *igt* were more increased in pyloric ceca than in head kidney.

4. Discussion

To the authors' knowledge, this is the first report describing the extensive transcript profile induced by an oral DNA vaccine [1] in any fish and against the infectious pancreatic necrosis virus (IPNV).

Table 2
List of primer pairs designed for gene expression analysis by RTqPCR.

Genes	Primer name	Primer sequence 5′–3′	Accession number
ifn1	IFN-1F	AAAACTGTTTGATGGGAATATGAAA	NM_001124531
	IFN-1R	CGTTTCAGTCTCCTCTCAGGTT	
ifng	IFNG-F	CTGAAAGTCCACTATAAGATCTCCA	FM864345.1
	IFNG-R	CCCTGGACTGTGGTGTCAC	
mx1	Mx1-F	AGCTCAAACGCCTGATGAAG	NM_001171901
	Mx1-R	ACCCCACTGAAACACACCTG	
mx3	Mx3-F	AGCTCAAACGCCTGATGAAG	U47946.3
	Mx3-R	TGAATATGTCTGTTATCCTCCCAAA	
stat1	stat1-F	TTGAGAGCATCGACTGGGAAAA	U60332.1
	stat1-R	GGCTAGGAGGTCATGGAAACGT	
mhc1	mhc-F	GCAACCCAATTTCATGCAGG	EU036638.1
	mhc-R	ACACTCAATGCAGGTCTGGG	
tnf2	tnf-F	TGCTGCTCCATGTGTGGTGC	DQ218473.1
	tnf-R	AGGGACGGGGAGCCTTGAT	
il8	IL-8F	GAATGTCAGCCAGCCTTGTC	AJ279069
	IL-8R	TCCAGACAAATCTCCTGACCG	
il10	IL-10F	CGACTTTAAATCTCCCATCGAC	AB118099
	IL-10R	GCATTGGACGATCTCTTTCTT	
il11	IL-11F	TGCGCTGCAGAGGAGCAAGT	AJ535687.1
	IL-11R	TGCTGGAGACCCCAAGCACA	
il12	IL-12F	ATGTGGTTACGGGAGGC	AJ548830.1
	IL-12R	ATGTGGTTACGGGAGGC	
bdef1	db-1F	GTTGTGGAGGATGAGGCTGC	FN545575.1
	db-1R	TGGCAGGCAAAGTTTTCGG	
igm	IgM-F	ACCTTAACCAGCCGAAAGGG	X65263.1
	IgM-R	TGTCCCATTGCTCCAGTCC	
igt	IgT-F	AGCACCAGGGTGAAACCA	AY870265
	IgT-R	GCGGTGGGTTCAGAGTCA	
ef1	EF-F	GATCCAGAAGGAGGTCACCA	AF498320
	EF-R	TTACGTTCGACCTTCCATCC	

Both internal immune responsive organs (head kidney) as well as some of the tissue first exposed to the vaccine (pyloric ceca) were analyzed. A 60-mer oligo microarray enriched in rainbow trout immune-related genes (2.5-fold with respect to previous designs) was used. The use of a microarray enriched in immune-related genes present in the GenBank, in addition to those probes previously obtained from EST sequences [17], not only allowed the study of a larger number of rainbow trout immune-related genes but also a high reliability of the data because of their exact annotation. Similar designs could be made with any other fish species for which sufficient gene numbers are deposited in the GenBanks (i.e. salmon, zebrafish, etc).

Seven days after oral vaccination was chosen because previous evidence of VP2 transcript expression in head kidney, pyloric ceca or other organs after oral [1] or intramuscular [37] DNA vaccination. That allows us to evaluate trout transcript expression during VP2 expression.

In orally vaccinated trout, the number of increased immune-related transcripts differentially expressed was higher in head kidney than in pyloric ceca. In contrast, the number of differentially decreased transcripts was higher in pyloric ceca than in head kidney. Those levels were confirmed by comparing absolute immunofluorescence measurements (Fig. 1), by comparing differentially expressed transcripts with folds >2 (Fig. 2), by individually studying the genes with folds >4 and $p < 0.05$ (Supplementary data) or by analysis of the individual gen groups with folds >2 (Figs. 3–8). All those results demonstrate that 7 days after vaccination the transcript response is more intense in head kidney (an internal immune response organ) than in pyloric ceca (one of the hypothetical entrance sites of the oral vaccine). Most probably some molecular signals had traveled from pyloric ceca to head kidney through the blood to induce such a response. Alternatively, pyloric ceca immune responsive cells might have migrated to the internal organ, thus causing a decreasing level of most transcripts in pyloric ceca. We have yet no evidence for either case.

Most of the studies of transcriptional responses in salmonid fish to IPNV infection that have been made by RTqPCR in Atlantic salmon [38–41] or cell culture [42] demonstrated induced expression of *ifn*-*mx*-related genes. For instance, both *type 1 ifn* and *ifng* have been detected and shown antiviral activity on IPNV [43]. With regard to trout intramuscular DNA vaccines to IPNV, we have previously described the induction of *in vitro* antiviral activity of pcDNA-VP2 and the *in vivo* stimulation of some immune-related genes [11]. Similar results were described for expression of *mx* and *ifn* genes in orally vaccinated trout [1] and confirmed in the present work with more genes. Thus, VP2 orally vaccinated trout increased several *ifng*-inducible genes in head kidney or pyloric ceca probably as a result of the increased *ifns*, as suggested in head kidney by *type 1 ifn*1 and in pyloric ceca by several *ifng* (*type 2 ifn*) transcript levels (Fig. 3). Other *type 1 ifns* (a, 3 and 4) were inhibited in head kidney, but their functional characterization is not yet available to make a possible interpretation [44,45]. Recent results

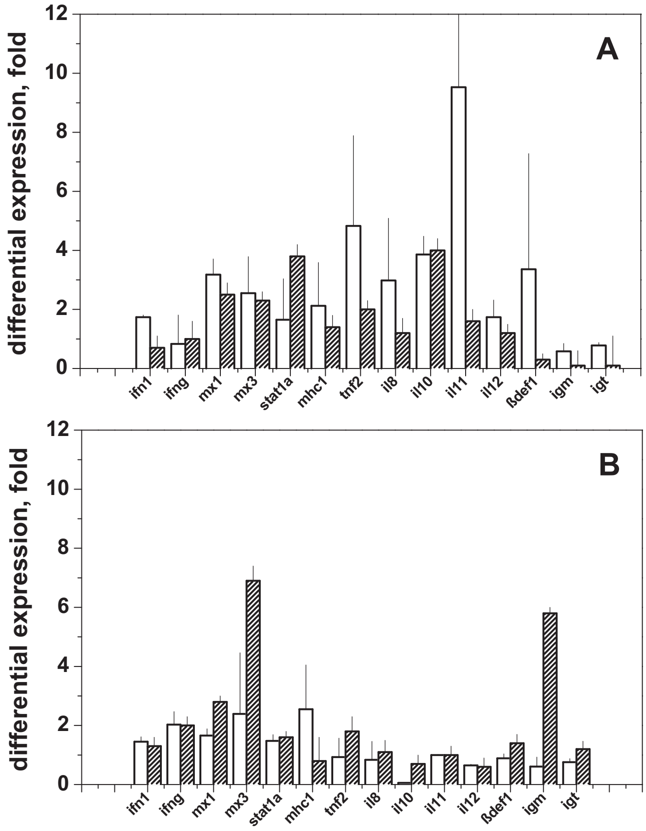

Fig. 9. Comparison between differential expression levels of selected transcripts obtained by hybridization to microarray (empty bars) and RTqPCR (hatched bars) in head kidney (A) and pyloric ceca (B). The RNA samples were the same than those used for the microarray. The cDNA were obtained, and qPCR performed as indicated in Section 2 by using SYBR green. The elongation factor 1α (*ef-1α*) gene was used to normalize the results. All qPCR reactions were performed in triplicate and the results were expressed as mean ± standard deviation. The expression levels were calculated as relative folds of the expression of pcDNA controls according to the $2^{-\Delta\Delta CT}$ method [18]. *ifn1*, type 1 interferon 1; *ifng*, type II interferon gamma; *mx1* and *3*, interferon-inducible mx proteins 1 and 3; *stat1*, transcription factor participating in interferon pathways; *mhc1*, major histocompatibility complex I molecules; *tnf2*, tumor necrosis factor 2; *il8*, CXC chemokine involved in neutrophil chemotaxis; *1l10*, interleukin which inhibits cytokines such as *ifng*, *il2*, *il3*, *tnf* and *csf*; *1l11*, interleukin inhibitor of apoptosis; *1l12*, interleukin that has been tested as vaccine adjuvant; *βdef1*, beta defensin 1 antimicrobial peptide; *igm*, serum immunoglobulin M; and *igt*, mucosal immunoglobulin T.

suggest complementary roles of all viral-induced *ifns* [46], which are in the same direction than the results commented above.

All the *ifn*-inducible *mx1*, *mx3* (cytoplasmic localization) and *mx2* (nuclear localization) transcripts characterized in rainbow trout [25,47] increased in head kidney while only *mx3* increased (Fig. 4 up) in pyloric ceca (the site of delivery of the oral vaccine). Similar results were obtained after intramuscular vaccination. Thus, expression of the 3 *mx* isoforms were obtained in head kidney while only *mx3* increased in muscle (the site of delivery of the DNA vaccine) [48]. All these results suggest that at least in head kidney, the expression of all 3 *mx* isoforms might be required for their antiviral effects, as also described after rhabdoviral or IPNV infection of rainbow trout [48] or sea bream [49], respectively. Thus, the 3 fish *mx* isoforms might have complementary mechanisms to inhibit viral replication in internal organs. The differential expression levels induced in internal versus local organs, however, suggest different but yet difficult-to-understand functions for fish *mx* isoforms.

Pentraxin (an acute phase protein related to C-reactive protein) showed the highest elevated transcript levels (>30-fold) (Fig. 5A). Similar increased pentraxin transcript levels were reported before only after bacterial infections [50]. However, it is not known what component in the present DNA vaccine formulation triggers such an increased level of pentraxin and whether or not it is important for the vaccine success. On the other hand, it might be relevant that 3 different probes of *cd11* (a marker of dendritic cells) were increased in both head kidney and pyloric ceca, suggesting a role of dendritic cells (antigen-presenting cells) in the responses induced by oral vaccination. At this respect, other increased dendritic cell-associated transcripts were also detected: *tlr5m* (located in the membrane of dendritic cells recognizing bacterial flagellins), *tnf11* (dendritic survival factor), *cd209* (c-type lectin marker of dendritic cells), *vig1* (VHSV-induced gene in dendritic cells), and *il12* (produced in dendritic cells in response to intracellular pathogens).

Head kidney genes corresponding to the classic complement pathway and its membrane attack complex were increased, suggesting an activation of the classic complement pathway. Although both precursor and mature properdin transcripts (main actors of the alternative pathway) were also increased, the simultaneous upregulation of factor *H* suggests a possible inhibition of the alternative pathway in this organ. In contrast, properdin and perforin genes remained increased while the factor *H* gene together with the *c3*, *bf2*, *cfh*, and *cr* genes were decreased in pyloric ceca, thus suggesting that the alternative pathway might be more active in this organ (Fig. 5 down).

The small fold changes detected in both head kidney and pyloric ceca, on *tlr* members, suggested that those genes do not participate on the response to VP2 oral vaccination. Nevertheless, the membrane-bound *tlr5m* was induced in both organs and showed an opposite behavior to soluble *tlr5*. The possible functional meaning of those results is not yet known. With respect to the tumor necrosis factor (*tnf*) family of pro-inflammatory cytokines, many changes were induced in *tnf*-related gene transcripts (9 probes with folds >2) in head kidney during oral VP2 vaccination (Fig. 6 down), similarly to what has been described for *tnf*α and *tnf*β during IPNV infection in fish cells [40].

Catfish-like chemokine *scya109* was increased after VP2 vaccination in head kidney while *scya113* was decreased. Both *scya109* [51] and *scya113*[52] increased after bacterial infection in catfish but there is no more functional information available in any other fish. Similarly to IPNV infection [53,54] and rhabdoviral DNA vaccination [30], VP2 oral immunization also increased other chemokines such as *ck5*, *ck6*, *ck7*, *ck9* and *ck12*.

The high number of interleukins (*ils*) showing increased transcription in head kidney suggests that this organ is at its optimum immune response 7 days after vaccination (Fig. 7 down). Thus, increased expression of pro-inflammatory cytokines such as *il8*, *il11* and *il12* was observed in head kidney although *il1*β transcripts were down-regulated in pyloric ceca. IPNV/IHNV infections increased expression of *il1*β and *il18* during single infections but decreased *il1*β expression after co-infection in brown trout (*Salmo trutta*) [55,56]. Down-regulation of *il1*β has also being correlated with chronic inflammation in salmon [57]. The suppression of *il1*β expression observed in pyloric ceca in this work may, therefore, indicate an inhibition of earlier inflammatory processes.

Increased *il12* transcripts were found in head kidney (Fig. 9A). Mammalian *Il12* stimulates *tnfa*, and several *tnf* were also increased in head kidney in orally vaccinated trout (Fig. 6 down). *Il12* has been described as a viral vaccine adjuvant for mucosal immunity [58] but it has not been tested in fish. Other transcripts, such as *il17* (Fig. 7 down) also increased their levels in head kidney. Since *il17* helps to induce antimicrobial peptides [59,60] that might explain why antimicrobial peptides were also increased in head kidney (*leapa2* and several β*defs*) (Fig. 8 up) and pyloric ceca (β*def*/β*def*). Because previous findings have implicated β*def* in both rhabdoviral blocking and activation of trout immune defense genes [61,62], antimicrobial peptides such as *leapa2* and β*defs* might deserve further studies.

The increase in differential transcription of several heat shock proteins (*hsp70*, *hsp40* and *hsp90)* together with prefoldin (*pfd*) in head kidney indicated that some new protein(s) were being translated after vaccination. The increase in 12 different probes of cathepsin (*cath*) proteases (family of related lysosomal proteases implicated in antigen processing) also suggests an activation of antigen processing due to the appearance of new protein(s) such as those probably derived from the transcription of the pcDNA-VP2 plasmid [1].

The increased *igm* and *igt* transcript expression levels detected in pyloric ceca compared to kidneys (Fig. 9B), might be important because of their potential implication on mucosal immunity, however, those increased levels were only detected by RTqPCR.

The data obtained is very consistent because it was obtained from four different populations of rainbow trout, and each population sample was obtained by pooling 6 trout. These results obtained 7 days after oral vaccination, demonstrate that the 30-day protection induced by these vaccines [1] was preceded by a complex and specific immune response in internal as well as in local organs. However, to understand the factors affecting expression of these different genes requires further work. Our present results indicated that series of time-course experiments in different organs focused in some of the immune-related genes identified in this work could be the next step to provide a more detailed view of these processes.

GenBank nucleotide sequences were searched with the following keywords: interferon, chemokine, interleukin, cytokine, defensin, macrophage, lymphocyte, antimicrobial, neutrophil, leukocyte, cytotoxic, natural killer, antiviral, antibacterial, LPS, Vig, antigen, histocompatibility, phagocyte, viral, Mx, complement, immunoglobulin, hepcidin, IgG, IgM, Toll, T cell, B cell, dendritic, presenting, TNF, perforin, MHC, NK, transcription, chaperone, stress, Hsp, Hsp70, Hsp90, tlr, flagellin, keratinocyte, cathepsin, NOD, IRF, IKK, JBK1, TRAM, TAK, TAB, JNK, P38, AP-1, TIRAP, IgT, IgH, high mobility. The resulting list of genes (GenBank sequences) was submitted to Agilent's eArray designing tool (https://earray.chem.agilent.com/earray/search.do?search1/4arrayDesign) to retrieve the Agilent list. The unique 60-mer probes were then added to the immune-related probes similarly extracted from the previously published EST-derived oligo array plus 3336 unknowns (Previous ID16271) [17] and classified into 16 groups to facilitate analysis. The final randomly arranged 8x15K Agilent's design (ID32303) was 2.5-

fold enriched in immune-related sequences, with a total of 6442 different sequences each by duplicate.

Acknowledgments

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2012.03.016.

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Una vacuna eficaz podría imitar inicialmente la acción del virus para inducir en el hospedador las respuestas defensivas necesarias. Por ello nos propusimos conocer si existe una similitud o mimetización en la respuesta inmune temprana, intermedia y tardía similar en los peces vacunados con pcDNA-VP2 por vía oral y en los peces infectados con IPNV. A diferentes días, se comparó la expresión de genes inmunológicos es en truchas vacunadas con pcDNA-VP2 o infectadas con IPNV.

1.2. Comparación de la respuesta transcripcional inmune en órganos de truchas vacunadas oralmente con pcDNA-VP2 y en truchas infectadas con IPNV.

Diseño experimental:

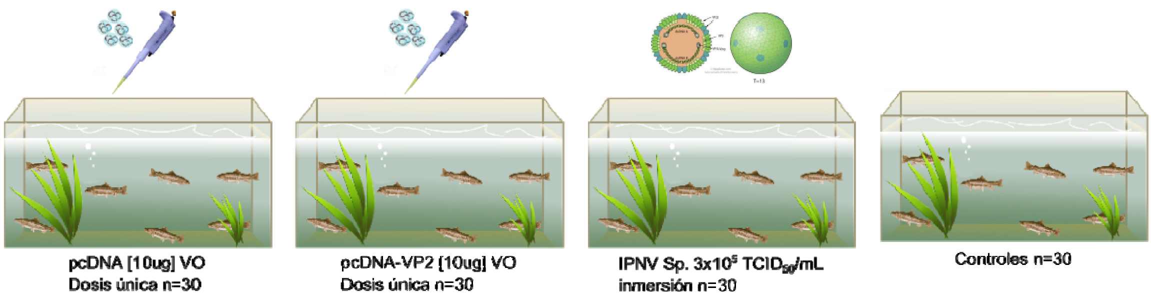

Figura 1: Diseño experimental

Alevines de trucha arco iris de aproximadamente 1 g y 3.5 cm de longitud.

- Grupo de peces (n=30) vacunados con 10 μg de pcDNA-VP2 vía oral "vacunados".
- Grupo de peces (n=30) vacunados con 10 μg de pcDNA vía oral "plásmido vacío".
- Grupo de peces (n=30) sin tratamiento "Controles"
- Grupo de peces (n=30) infectados con IPNV. se infectaron con 3×10^5 $TCID_{50}mL^{-1}$ de IPNV Sp durante 2 horas por inmersión (n=30) "Infectados"

Tiempos de muestreo: En peces vacunados 0, 2, 3, 5, 7, 10 y 15 días post-tratamiento. En peces infectados 0, 2, 3 y 5 días post-infección (n=3 peces en cada muestreo).

Muestras: riñón anterior, bazo, intestino posterior, ciego pilórico y timo.

Métodos: Extracción de RNA total utilizando TriZol, síntesis de cDNA, PCR cuantitativa a tiempo real. Los genes evaluados fueron: STAT1, IFN-I, IFNγ, Mx1, Mx3, IL8, IL10, IL11, IL12b, TNF2, MHC-I, IgM e IgT.

Resumen:

Los resultados mostraron que la vacunación con pcDNA-VP2 por vía oral imitó los perfiles de transcripción obtenidos tras la infección con IPNV, tanto en niveles de expresión y de tiempo en los órganos seleccionados.

Por otra parte, los niveles de expresión diferencial más altos se obtuvieron en el timo, lo que sugiere que este tejido podría ser importante para la inducción de la protección contra la infección. Sin embargo, los niveles de expresión diferencial de la mayoría de los genes fueron menores en los peces vacunados respecto a los peces infectados, a excepción del IFN-I que alcanzó niveles de transcripción similares en ambos grupos. Estos resultados mostraron que pese a que la vacuna genera una estimulación de genes de respuesta inmune similar y con el mismo perfil al observado ante la infección viral, no presenta los mismos niveles, por lo que cabe sugerir que todavía se pueden encontrar métodos o mecanismos para mejorar el rendimiento y la protección de la vacuna, como puede ser otros tipos de recubrimiento.

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Trout oral VP2 DNA vaccination mimics transcriptional responses occurring after infection with infectious pancreatic necrosis virus (IPNV)

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ABSTRACT

Time-course and organ transcriptional response profiles in rainbow trout *Oncorhynchus mykiss* were studied after oral DNA-vaccination with the VP2 gene of the infectious pancreatic necrosis virus (IPNV) encapsulated in alginates. The profiles were also compared with those obtained after infection with IPNV. A group of immune-related genes (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *il11*, *il12b*, *tnf2*, *mhc1uda*, *igm* and *igt*) previously selected from microarray analysis of successful oral vaccination of rainbow trout, were used for the RTqPCR analysis. The results showed that oral VP2-vaccination qualitatively mimicked both the time-course and organ (head kidney, spleen, intestine, pyloric ceca, and thymus) transcriptional profiles obtained after IPNV-infection. Highest transcriptional differential expression levels after oral vaccination were obtained in thymus, suggesting those might be important for subsequent protection against IPNV challenges. However, transcriptional differential expression levels of most of the genes mentioned above were lower in VP2-vaccinated than in IPNV-infected trout, except for *ifn1* which were similar. Together all the results suggest that the oral-alginate VP2-vaccination procedure immunizes trout against IPNV in a similar way as IPNV-infection does while there is still room for additional improvements in the oral vaccination procedure. Some of the genes described here could be used as markers to further optimize the oral immunization method.

1. Introduction

Infectious pancreatic necrosis (IPN) is an economically important fish disease caused by a virus belonging to the *Birnaviridae* family which produces severe acute infections in young salmonids (i.e.: salmon and trout) and many other fish, resulting in the most widespread virus in aquaculture [1,2]. Furthermore, following a disease outbreak, surviving fish may become asymptomatic carriers contributing to the spreading of the disease. Therefore, broodstock carriage is considered a source of virus for the lethal infection of hatchery-reared fry. The development of an effective vaccine will be a convenient way to secure the future of fish farms and the health status of both inland and sea waters.

Because present oil-adjuvanted vaccines based on recombinant IPNV proteins delivered by intraperitoneal injection have important side effects on fish welfare [3–5], present licenced DNA vaccines still require fish-to-fish intramuscular injection [6] and small immunocompetent fish cannot be injected, oral vaccines are an important alternative to immunize against IPNV. However, oral vaccines have many requirements that are difficult to obtain, such as their need to be protected from stomach digestion, adhere to the fish guts, avoid induction of immune tolerance, induce the appropriated immune protective responses and be capable of being mixed with the feed. Thus although oral delivery has been considered the most desirable way to vaccinate both humans and animals [7,8], despite many research efforts there are yet few reports describing successful methods. Nevertheless, recent reports using pathogen recombinant proteins in salmon [9,10] and DNA in rainbow trout [11] or in Japanese flounder (*Paralichthys olivaceus*) [12], now suggest fish oral vaccination might be possible. However, the knowledge of immune-related responses to improve fish oral vaccines is still preliminary [13].

Intramuscular DNA vaccines can induce strong and long-lasting humoral and cell-mediated fish immune responses, resembling those induced by natural infection of intracellular pathogens [10,14]. In contrast, oral DNA vaccines are just beginning to be tested in fish in a similar way and therefore, little is known about the significance of the up or down regulation of the differential expression of genes related to antiviral activity [13]. Because, the knowledge of the transcriptional responses in salmonid fish to

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IPNV-infection is often limited by the small number of immune-related probes available [15], most of the studies have been developed in Atlantic salmon by RTqPCR demonstrating induced differential expression of several interferon-related genes [16]. More recently, up-regulation of *ifn* and *mx* genes together with inflammatory and proteolysis genes in fish survivors to IPNV lethal challenges have been also reported [17]. With regard to IPNV intramuscular DNA vaccines, the VP2 gene stimulation of some immune-related genes in rainbow trout has been described [18]. Furthermore, protection levels up to 83% RPS (relative percentage survival), neutralizing antibodies lasting for at least 60 days and early differential expression of *ifn* and *mx* transcripts was obtained by using VP2 complexed with alginates in orally vaccinated trout [11]. Finally, by combining EST-derived trout oligo-probes [19] with oligo-probes designed from selected GenBank gene entries, we used a newly designed oligo-microarray enriched in immune-related *Oncorhynchus mykiss* genes to study early transcript differential expression after oral vaccination with the VP2 gene [13]. Seven days after oral vaccination, a wide list of vaccine-upregulated genes in head kidney compared to pyloric ceca could be thus identified. A group of selected genes from that study was selected to carry out the present studies.

This work describes the time-course and organ profiles of selected transcriptional responses after oral DNA vaccination of rainbow trout *O. mykiss* and compares those to the corresponding profiles obtained after infection with IPNV. The IPNV VP2 gene encoded into a plasmid DNA and encapsulated in alginate microspheres [11] and a group of immune-related rainbow trout genes (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *il11*, *il12b*, *tnf2*, *mhc1uda*, *igm* and *igt*) selected as mentioned above [13] and their differential expression estimated by RTqPCR, were used for the comparisons. The results showed that the oral VP2-vaccine could mimic both the time-course (up to 7 days) and the organ (head kidney, spleen, intestine, pyloric ceca, and thymus) profiles of transcripts obtained after IPNV-infection. However, transcriptional levels were lower in VP2-vaccinated than in IPNV-infected trout, except for *ifn1* which were similar. The results obtained suggest that the mechanisms by which alginate-oral DNA vaccination induces protection are similar to the defense mechanisms induced by IPNV-infection. Furthermore, some of the genes described here could also be used as markers to follow up immunization procedures and/or to further optimize oral vaccination.

2. Materials and methods

2.1. Preparation of the IPNV VP2 DNA plasmid vector

The plasmid DNA was prepared as described previously [11]. Briefly, the IPNV VP2 gene was cloned into the pcDNA3.1/V5/His-TOPO (Invitrogen, Spain) plasmid vector under the control of the immediate-early CMV promoter (pDNA-VP2 or VP2-vaccine) and amplified in *Escherichia coli* TOP10. The plasmid DNA was isolated with the Endofree Plasmid Maxi purification Kit (Qiagen Iberia, S.L.-Spain) according to the manufacturer's instructions. The DNA concentration was measured in a spectrophotometer (NanoDrop 2000, Thermo scientific, Spain) before it was aliquoted and conserved at −20 °C. The pcDNA3.1/V5-His-TOPO plasmid (pDNA) was used as control plasmid.

2.2. Preparation of microspheres and formulation of the oral VP2-vaccine

The procedure was followed as previously described [11]. Briefly, 2.5 mL of 3% (w/v) of sodium alginate were mixed with 1.5 mL of 1 mg/mL of plasmid pDNA-VP2 and the mixture stirred at 500 rpm during 10 min. This solution was then added to an Erlenmeyer flask containing 100 mL of paraffin oil and 0.5 mL Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared by adding 2.5 mL of 0.15 M $CaCl_2$ to the emulsion drop by drop and stirring for 2 h at 900 rpm. Microspheres were collected by centrifugation at 1000 *g* for 10 min, and they were washed twice with 70% ethanol, lyophilized for 24 h and stored at 4 °C.

2.3. Experimental fish: rainbow trout

Rainbow trout (*O. mykiss*) of a mean weight of 1 g (mean size of 3.5 cm) were purchased from a spring water local farm with no history of viral disease. No fish showed any clinical signs. In addition, two pools of 5 fish each were tested by standard methods to confirm the absence of IPNV or any other salmonid virus by isolation using BF cells [20]. The trout were acclimatized for 2 weeks and kept under a 12/12 h light/dark regime at 15 °C in 350 l closed re-circulating water tanks (Living Stream, Frigid Units Inc, Ohio) at the “Centro de Investigaciones Biológicas” (CSIC, Madrid, Spain). Groups of trout were maintained in separate 45 l aquaria supplied with non-chlorinated water using exterior carbon filters (Eheim, Madrid, Spain) and additional aeration. The trout were fed daily with a diet of commercial pellets. The water-quality parameters were maintained at optimum levels for rainbow trout and the culturing conditions in all tanks were equal. Experimental protocols were performed with the approval of the CSIC ethical committee.

2.4. Oral VP2-vaccination

The trout were divided into three groups of 30 trout each. A group of trout was orally vaccinated with 10 μl of suspension of the vaccine microspheres each containing 10 μg of pDNA-VP2 diluted in 10 μl of PBS, while other group received similar microspheres suspension but with pDNA (empty plasmid). An untreated fish group was maintained unhandled. Vaccination was performed with an automatic pipette with a 20 μl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the oesophagus. All fish were sacrificed by exposing to an overdose of tricaine methane sulphonate (MS-222, Sigma, Madrid, Spain) prior to tissue sampling. Head kidney, spleen, intestine, pyloric ceca and thymus from each trout were collected at 2, 3, 5, 7, 10 and 15 days post-vaccination and stored in TRIzol LS reagent (Invitrogen, Spain) until RNA isolation. There were 3 trout for each of 6 time points. In addition, 3 trout were sacrificed prior to IPNV immersion challenge, and their tissue samples collected, to serve as the time 0 h control. Because of their small size and similar location of the thymus and pseudobranch tissues, thymus harvested for the analysis was mixed with pseudobranch tissue. As estimated by fluocytometry (SSC/FSC) and confirmed by smear staining, the thymus tissue contained 19–20.6% of lymphocytes, the rest being red blood cells and large pseudobranch cells (i.e.: chloride cells) [21].

2.5. IPNV challenge by immersion

The trout were divided into 2 groups of 25 trout each. Group 1 was infected with IPNV in a reduced volume of water for 2 h with aeration (3×10^5 tissue culture infectious dose $TCID_{50}$/ml of IPNV). Group 2 was handled similarly but was mock infected. Head kidney (HK), spleen (S), intestine (IN), pyloric ceca (PC) and thymus (T) from each fish were collected at 2, 3, 5, and 7 days post-challenge. Since mortalities began after 7 days, there were no time points after 7 days. Samples were stored in TRIzol LS reagent until RNA isolation. There were 3 trout for each of the time points.

2.6. Isolation of total RNA and cDNA synthesis

Organs were individually homogenized in 1 mL of TRIzol using the Tissue Lyser Cell Disruptor (Qiagen S.A, Spain) 5 min at 50 Hz with 2 mm glass beads. Total RNA was extracted by the TRIzol reagent according to the manufacturer's instructions, and the concentration and purity of the RNA obtained were measured in a NanoDrop™ spectrophotometer. RNA extracted was resuspended in pyrogen free DEPC treated water. RNA was treated with DNase I RNase free (Fermentas, Spain). The cDNA synthesis was performed with 5 μg of the RNA primed with oligo-d(T) (25 pmol/μl). The Super Script™ II kit (Invitrogen, Spain) was used for reverse transcription. The cDNA was diluted to 1:4 in DEPC treated water and 1 μl of the diluted cDNA was taken for each reaction in the real-time PCR assay.

2.7. Quantitative estimation of transcripts for selected immune-related genes by qPCR

The qPCRs (quantitative polymerase chain reactions) were performed by using the SYBR® green method, in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories, Inc., Madrid, Spain). The qPCR amplifications were carried out in 96-well plates by mixing 1 μl of 4-fold diluted cDNA, 12.5 μl of 2× concentrated iQ SYBR® Green Super mix (Bio-Rad), 0.3 μM forward primer and 0.3 μM of reverse primer in a 25 μl reaction volume for each sample. The thermal profile was 10 min at 95 °C, followed by 40 amplification cycles of 10 s at 95 °C, 1 min at 60 °C and a dissociation cycle (1 min at 95 °C and 1 min at 60 °C). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. No amplification product was observed in controls containing no RNA samples. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and the relative quantification of the amplified gene products was calculated by the comparative Ct method. The elongation factor 1α (*ef1a*) was used as the house keeping gene in each RNA sample in order to normalize the results by eliminating variation in mRNA and cDNA quantity and quality (normalized values). All the qPCR reactions were performed in duplicate (technical replicates) and their mean Ct values used for the calculations. First, the Ct for each gene was normalised to their corresponding *ef1a* Ct ($\Delta Ct_{gene} = Ct_{gene} - Ct_{ef1a}$). Second, mean control Ct values (mean $\Delta Ct_{control}$) from 3 trout immunized with empty plasmid for the VP2-vaccination or mock infected for the IPNV-infection were calculated. Third, folds were calculated by the $2^{-\Delta\Delta Ct}$ method [22], where $\Delta\Delta Ct_{gene} = \Delta Ct_{gene} -$ mean $\Delta Ct_{control}$. Finally, the mean and their standard deviations for each gene from 3 trout were calculated and represented. When ratios between IPNV-infected and VP2-vaccinated trout values were calculated (i.e.: Fig. 4), the corresponding standard deviations were derived by following the formula, square root of the sum of the squares of each of their standard deviations (User Bulletin #2: ABI PRISM 7700 sequence detection system, PE Applied Biosystems).

2.8. Statistical analysis

Prior to statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro–Wilk test. Data are presented as mean ± standard deviation of 3 trout. Factorial ANOVAs were run to determine if the differential expression gene differed between the replicates at an individual gene followed by Tukey's multiple comparison test for differences between the vaccinated group and IPNV-infected group. The Student *t* test was used also to compare some paired samples. All statistics were run in SPSS Version 15. The *p* value which was less than 0.05 was considered to be significant.

3. Results

3.1. Selection of trout genes

To compare the differential expression of transcripts induced by the oral VP2-vaccination with those induced by IPNV-infection, some of the genes previously found with differential expression >2-fold in oral VP2-vaccination and/or induced during IPNV-infection of trout were chosen. The genes selected could be distributed in 3 groups, (i) those belonging to interferon-related pathways such as *stat1* (an intermediary *ifn* responsive transcriptional protein) [23], *ifn1* [24], *ifng* [25], *mx1* [26] and *mx3* [27–29], (ii) those related to cytokines/interleukines such as *il8* [30], *il10* [31], *il11* [32], the p40 b chain of the *il12* heterodimer [33] and *tnf2* (similar to *tnfa*) [34] and (iii) those related to adaptive immune responses such as *mhc1uda* [35], *igm* [36] and *igt* [37]. The *mhc1* corresponding to the α chain sequence of the UDA-HC allele was chosen because previous microarray data showed to be regulated in most trout of the population used for the assay [13]. Table 1 shows the corresponding primer sequences designed for the RTqPCR analysis of those genes.

3.2. Time course of transcript differential expression after oral VP2-vaccination

To select for the optimal time to make the organ comparisons and to study whether the changes in differential transcript levels after VP2-vaccination were time-dependent, those were comparatively studied in head kidney (HK) and thymus (T) at different

Table 1
List of primer pairs designed for gene expression analysis by RTqPCR.

Genes	Primer sequence 5′–3′: forward reverse	Accession number
Interferon -related		
stat1	TTGAGAGCATCGACTGGGAAAA GGCTA GGAGGTCATGGAAACGT	U60332.1
ifn1	AAAACTGTTTGATGGGAATATGAAA CGTTTCAGTCTCCTCTCAGGTT	NM_001124531
ifng	CTGAAAGTCCACTATAAGATCTCCA CCCTGGACTGTGGTGTCAC	FM864345.1
mx1	AGCTCAAACGCCTGATGAAG ACCCCA CTGAAACACACCTG	NM_001171901
mx3	AGCTCAAACGCCTGATGAAG TGAATAT GTCTGTTATCCTCCCAAA	U47946.3
Cytokines /interleukines		
il8	GAATGTCAGCCAGCCTTGTC TCCAGAC AAATCTCCTGACCG	AJ279069
il10	CGACTTTAAATCTCCCATCGAC GCATT GGACGATCTCTTTCTT	AB118099
il11	TGCGCTGCAGAGGAGCAAGT TGCTGGA GACCCCAAGCACA	AJ535687.1
il12b	ATGTGGTTACGGGAGGC ATGTGGTTA CGGGAGGC	AJ548830.1
tnf2	TGCTGCTCCATGTGTGGTGC AGGGACG GGGAGCCTTGAT	DQ218473.1
Adaptive response		
mhc1uda-hc	GCAACCCAATTTCATGCAGG ACACTCA ATGCAGGTCTGGG	EU036638.1
igm	ACCTTAACCAGCCGAAAGGG TGTCCCA TTGCTCCAGTCC	X65263.1
igt	AGCACCAGGGTGAAACCA GCGGTGGG TTCAGAGTCA	AY870265
House keeping gene		
ef1a	GATCCAGAAGGAGGTCACCA TTACGT TCGACCTTCCATCC	AF498320

times after vaccination. Fig. 1 shows that differential transcript levels in T were higher than in HK at all the time points studied, for most of the selected genes except for *ifn1* which showed a similar evolution in both organs.

Not only the magnitude but also the timing of transcript induction was different in HK and T. Thus, in HK, the VP2-vaccination induced significant increases in *ifn1* gene differential expression from the 3rd to the 10th day post-vaccination, peaking at day 7 with values of ~45 fold. The *ifn1*-related genes *mx1*, *mx3*, and *stat1* had smaller but significant increased differential expression levels, <10 fold and occurring between 5 and 7 days post-vaccination (Fig. 1A). Interestingly, differential expression of *ifng* was also detected early and up-regulated from 5 to 10 days post-vaccination although with ~8–9 folds. The interleukines and the rest of genes except *igt* were also detected and

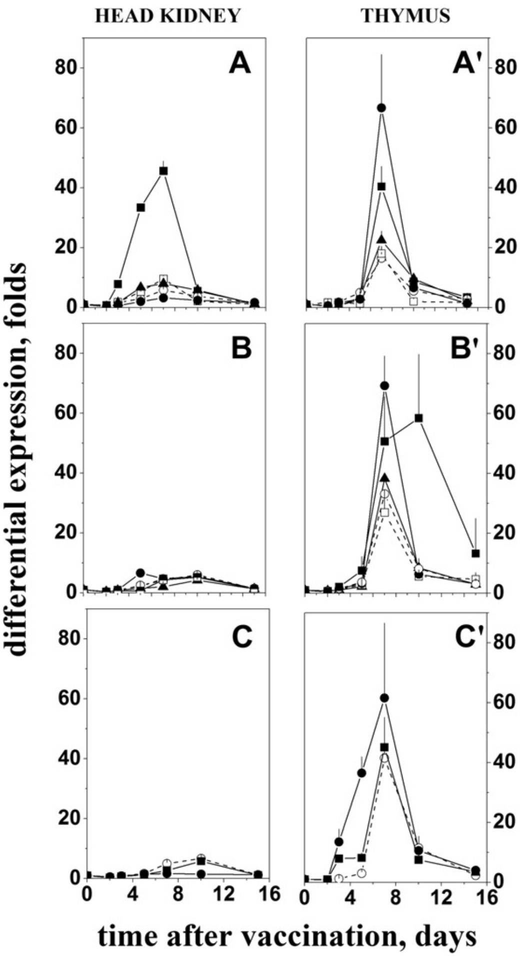

Fig. 1. Comparison of time-course of differential expression folds of selected genes in trout head kidney (HK) and thymus (T) after oral VP2-vaccination. Rainbow trout were vaccinated with pDNA-VP2 or mock vaccinated with pDNA (methods). The gene differential expression was evaluated in HK and T during 2, 3, 5, 7, 10 and 15 days after immunization. The primers described in Table 1 were used for the RTqPCR as described in methods. The differential expression of target genes was normalised to the endogenous *ef1a* gene and folds were then calculated by the $2^{-\Delta\Delta Ct}$ method. The figure shows the mean and standard deviations (sd) of folds from different trout ($n = 3$). Only the maximal sd was drawn for clarity. When compared to time 0, most of the data were significantly different at the $p < 0.05$ level, except for all genes after 2 and 15 days, *mx3* at day 3, *igt* at days 5 and 10 and *il11* and *il12b* at day 3 in HK or for most genes after 2 days and *ifn1* and *mx3* at day 3 in T. A, A′, interferon-related genes: ●, *stat1*; ■, *ifn1*; ▲, *ifng*; ○, *mx1*; □, *mx3*. B, B′, cytokines/interleukins-related genes: ●, *il8*; ■, *il10*; ▲, *il11*; ○, *il12b*; □, *tnf2*. C, C′, adaptive response-related genes: ●, *igt*; ■, *igm*; ○, *mhc*.

showed 4–6-fold increase of differential expression from 5 to 10 days post-vaccination. Fifteen days post-vaccination, nearly basal levels of differential expression were observed for all the genes studied.

In T significant differences of transcript differential expression levels were recorded at the different time points for most of the genes. Differential expression of *ifn*-related genes began to increase at day 5 (2–5 fold), peaked on day 7 with values ranging from ~16 fold (*mx3*) to 67 fold (*stat1*) and declined at day 10 (2–8 fold, *mx3* and *ifn1*, respectively) (Fig. 1A′). The cytokines/interleukines-related genes showed high differential expression 7 days post-vaccination (27–60 fold), with changes elevating from the 5 day post-vaccination, declining at 10 day and remaining significantly increased by day 15 (3–4 fold). Exceptionally, only *il12b* had a higher fold increased differential expression on day 15 (~13 fold, Fig. 1B′ bold squares).

With respect to the adaptive response-related genes, their time-course differential expression was slightly different, as they began to increase earlier, at 3 days post-vaccination, peaked on day 7 (42- to 61-fold) and declined by day 15 to 3–4 fold values. The greatest up-regulation values recorded were 67-fold for *stat1* and 61-fold for *igt* (Fig. 1A′ and C′, respectively).

Maximal differential expression levels in both HK (Fig. 1A) and T (Fig. 1A′) were obtained 7 days post-vaccination for all the interferon-related transcripts. Maximal differential expression levels were obtained not only at 7 days post-vaccination for *il10* in HK and *il8*, *il11* and *tnf2* in T, but also at 10 days post-vaccination for *il8*, *il12b* and *tnf2* in HK (Fig. 1B) and *il12b* in T (Fig. 1B′). Finally, maximal differential expression levels were obtained 10 days post-vaccination for *mhc1uda*, *igm* and *igt* in HK (Fig. 1C) or 7 days post-vaccination for *igt*, *mx*, *ifn1*, *ifn g*, *stat* and *tnf* in T (Fig. 1C′). Therefore, 7 days post-vaccination was chosen as the optimal time to further study the organ differential expression profiles of the selected genes.

3.3. Differential expression of selected genes in trout organs 7 days post-vaccination

The distribution of differential transcript levels of the genes mentioned above was then studied in rainbow trout head kidney (HK), spleen (SP), intestine (IN), pyloric ceca (PC) and thymus (T), 7 days after oral VP2-vaccination.

The highest increased differential expression levels of all the organs studied were obtained in T in all the selected genes (16–70-fold) (Fig. 2, yellow bars). Only HK *ifn1* (Fig. 2, white bar) and IN *igt* (Fig. 2, hatched bar) showed >20-fold increased levels, while SP *stat1*, *il11*, *il12b*, *mhc1uda* and *igm* showed more than 10-fold increased levels. After the T, HK showed higher differential expression levels in most of the genes in the interferon group while SP showed higher differential expression levels in the cytokines/interleukines group. Most of the downregulated and/or unchanged transcripts (folds <2 and folds ±1.9, respectively), were found in either IN (*ifn1*, *ifng*, *mx1*, *mx3*, *il8*) or PC (*ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *tnf2*, *mhc1uda*) (Fig. 2).

3.4. Comparative kinetics of HK gene differential expression between IPNV-infected and VP2-vaccinated trout

To compare transcript differential expression levels in VP2-vaccinated trout with those in IPNV-infected trout, differential expression of the selected genes was first studied at different times after immunization. Because HK was a target organ of IPNV multiplication and also the main trout internal immunological responsive organ, it was chosen to comparatively optimize the time to study organ distribution of transcript levels up to 7 days (Fig. 3).

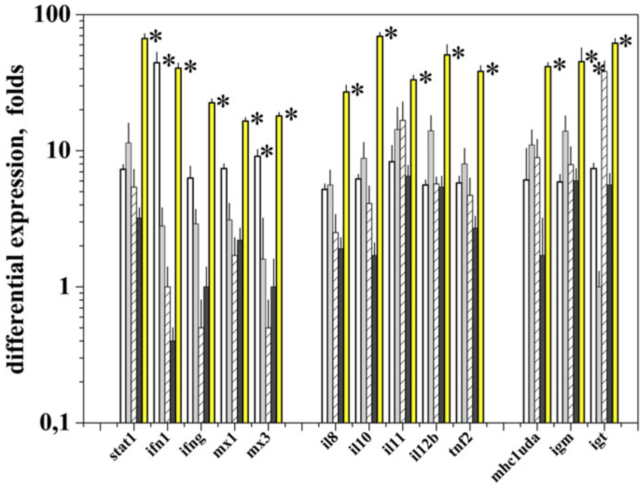

Fig. 2. Differential expression folds of selected genes in trout organs, 7 days after oral VP2-vaccination. Rainbow trout were vaccinated with pDNA-VP2 or mock vaccinated with pDNA. Organs were harvested 7 days after immunization. The trout selected primers described in Table 1 were used to estimate differential transcript levels in head kidney (HK) and spleen (SP) (internal organs) and pyloric ceca (PC), intestine (IN) and thymus (T) (surface exposed tissues). Assay conditions were as described in methods. The expression of target genes was normalised to the endogenous *ef1a* gene and folds were then calculated by the $2^{-\Delta\Delta Ct}$ method. The figure shows the mean and standard deviations of folds from different trout ($n = 3$). Only the maximal sd was drawn for clarity. HK, open bars. SP, light grey bars. PC, hatched bars. IN, black bars. T, yellow bars. *, gene folds >10 and significantly different to the rest of their corresponding organs at the $p < 0.05$ level.

Longer time points could not be studied because mortalities in the IPNV-infected group appeared after 7 days.

In HK from IPNV-infected trout, maximal levels increased with time up to more than 250-fold in *ifng* and *igm* transcripts while *ifn1*, *mx1*, *il8*, *il10*, *il12b*, and *igt* reached maximal levels in the 100–150-fold range. In the IPNV-infected trout, fold differential expression levels of around 10 or more were detected as early as 2 days for most of the selected genes, while in VP2-vaccinated trout, increased levels were most evident after 5 days. Although differences between transcript levels from IPNV-infected and VP2-vaccinated trout varied with time, differential expressions were generally 5–60-fold higher in IPNV-infected than in VP2-vaccinated trout except for *ifn1* which were only 2–3-fold higher, or for *igm*/*igt* which were 100–260/10–100-fold higher, respectively. Another exception was the peak differential expression of *il12b* in IPNV-infected trout after 5 days, which was almost 200-fold higher than in VP2-vaccinated fish. However, in all the genes studied, profiles after VP2-vaccination paraleled profiles after IPNV-infection. Because most of the data showed increasing levels of differential expression with time until reaching a plateau 5–7 days after immunization, 7 days was chosen to study their respective profiles of transcript levels in several organs.

3.5. Comparison of transcript expression of selected genes in different organs 7 days post-vaccination

Since we were interested in studying any differences between organs from either IPNV-infected or VP2-vaccinated trout, we first compared the levels of transcripts (values obtained after normalization) from either IPNV-infected or VP2-vaccinated trout. Since once normalized, the time profiles of HK expression levels (data not shown) were very similar to those of differential expression fold values (Fig. 3), we chose 7 days to make the comparison across different organs. Table 2 shows that IPNV-infected trout levels were up-regulated >6 fold in all studied organs for interferons (*ifn1* and *ifng*), *il8* and *igm*. Among the organs, HK showed the higher up-regulation, with normalized values >6 for *stat1*, *ifn1*, *ifng*, *mx1*, *il8*, *il10*, *il12b*, *mhc1uda*, *igm* and *igt* (all the studied genes except for *mx3*, *il11* and *tnf2*). Highest levels were 57.88–2304.12 for *il8* and up to 103.97 for *ifn1* or 236.39 for *igm*. In contrast, VP2-vaccinated trout transcript levels were less up-regulated than the corresponding IPNV-infected organs and only >30 for *il8* in all organs. Also, it was T, rather than HK, which showed levels >2 for *stat1*, *ifn1*, *ifng*, *mx1*, *il8*, *il12b*, *mhc1uda* and *igm* (all the studied genes except for *mx3*, *il11* and *tnf2* plus *il10* and *igt*). In this case, highest levels were 238.03 for *il8* and 45.25 for *ifn1* (Table 2).

We then compared the transcripts from IPNV-infected trout to those with mock-infected control on the one hand and from VP2-vaccinated trout with empty plasmid-vaccinated trout, on the other hand (differential transcripts or folds). Ratios between the folds from IPNV-infected and VP2-vaccinated trout were then calculated for each organ and plotted to make the comparison easier to follow. Thus, Fig. 4 shows that all transcript levels corresponding to interferon-related genes (*stat1*, *ifn1 ifng*, *mx1* and *mx3*) were 5–100-fold higher in IPNV-infected trout than in vaccinated trout in HK, SP or IN (all the internal organs). In contrast, all of those genes had lower or similar ratios in T or some of them in PC (external organs) (Fig. 4). Similarly, ratios of genes in the cytokines/interleukines group (*il8*, *il10*, *il11*, *il12b* and *tnf2*) or the adaptive response genes (*mhc1uda*, *igm* and *igt*) had higher ratios in HK, SP and IN and lower/similar ratios in T or some of them in PC (Fig. 4).

3.6. Transcript levels of VP2 in IPNV-infected and VP2-vaccinated trout

To help with the interpretation of the results obtained in IPNV-infected and VP2-vaccinated trout, the levels of the IPNV or the VP2-vaccine in HK were indirectly determined during several days by estimating the VP2 expression in each case. Time-course experiments revealed that in the IPNV-infected trout the level of VP2 peaked 7 days post-infection while in the VP2-vaccinated trout, the VP2 expression level was maintained from 2 to 15 days post immunization (results not shown). The VP2 expression induced by the IPNV-infection or the VP2-vaccine was also determined in organs 7 days after immunization or infection. Table 3 shows that depending on the organ, VP2 transcript expression was 10–100-fold higher in IPNV-infected trout than in VP2-vaccinated trout. Furthermore, VP2 transcript expression was highest in HK and IN than in the rest of the organs, while in VP2-vaccinated trout, VP2 levels were similar for all the organs.

4. Discussion

The results showed that in rainbow trout the oral VP2-vaccine encapsulated in alginate microspheres, could mimic most of the transcriptional profiles of differential expression induced by IPNV-infection both in organs and in timing. Not much was previously known concerning the organ distribution, degradation, persistence or responses after oral administration of pDNA since these vaccines are yet scarcely explored.

Several observations on the organ distribution of the plasmid DNA (pDNA) molecule had been reported for genetic vaccines after intramuscular administration. For instance, in Atlantic salmon, intramuscularly injected pDNA was transported into the blood plasma and was able to reach other organs (such as HK, SP and gills), suggesting that pDNA avoids degradation at the administration site and distributes through the internal and external organs [38]. It appeared that HK was preferentially acting as a scavenger tissue, clearing the pDNA from blood circulation. Our previous

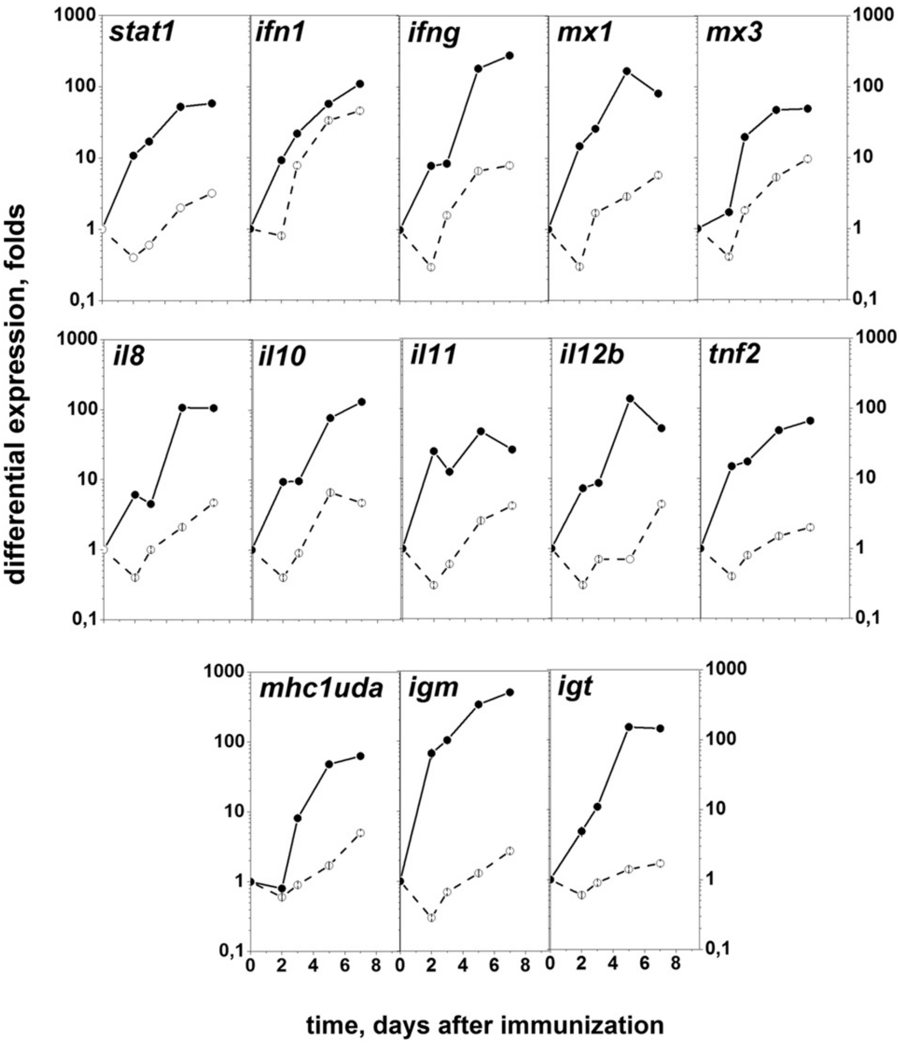

Fig. 3. Time course of differential expression folds of selected genes in head kidney (HK) from IPNV-infected and VP2-vaccinated trout. Assay conditions and analysis were as described in Fig. 1. The time course was followed up to 7 days, at which time mortalities appeared in the IPNV-infected group. The figure shows the mean and standard deviations of folds from different trout ($n = 3$). Only the maximal sd was drawn for clarity.●, HK transcripts from IPNV-infected trout; ○, HK transcripts from VP2-vaccinated trout. When compared to time 0, most of the data were significantly different at the $p < 0.05$ level, except for *mhc1uda* at 2 days.

results demonstrated that VP2 transcripts were expressed in different organs early after oral vaccination of trout with pDNA-VP2 (VP2-vaccine) encapsulated in alginate microspheres [11]. This oral vaccine induced a wide range of innate and specific immune responses, as well as a high level of protection [11,39]. The VP2 transcript expressed in organs after their passing through the digestive tract was able to stimulate numerous immune-related genes in HK and less in PC. To further study those effects, we selected several representative trout genes from our previous work to be further studied (*stat1*, *ifn1*, *ifng*, *mx1*, *mx3*, *il8*, *il10*, *il11*, *il12b*, *tnf2*, *mhc1uda*, *igm* and *igt*) by RTqPCR analysis of their kinetics and transcript changes in several trout organs (head kidney, spleen, intestine, pyloric ceca, and thymus) after oral VP2-vaccination. Differences of magnitude and timing of the corresponding transcripts were also determined for IPNV-infected trout.

One of the most significant findings for most genes was the unexpected high levels of transcripts induced after VP2-vaccination in thymus (T) compared to the rest of the organs (Fig. 2). T highest transcript levels could be explained because T should be one of the first tissues to come in contact with the VP2-vaccine even when the DNA is still tightly packaged by the alginate microspheres. However, VP2 expression was higher in IN and PC than in T after VP2-vaccination (Table 3), making the above mentioned explanation less likely. Alternatively, IN could be one of the first target tissues for the VP2-vaccine. In this former case, the T high transcript levels could be explained as responses to signals released from other VP2-targeted internal organs, such as HK and SP. Such hypothesis seems to be confirmed by the earlier transcript differential expression in HK (5 days) than in T (7 days), at least for *ifn1* (Fig. 1A) and *il10* (Fig. 1B).

Another important observation was that the time-course differential expression of most of the genes in HK in VP2-vaccinated trout paraleled those from IPNV-infected trout, although at 10–100-fold lower levels (Fig. 3). An exception to those differences was *ifn1*, which showed similar levels in IPNV-infected and VP2-vaccinated trout at all the time points. Would those *ifn1* levels be sufficient to get the high protection obtained by VP2-vaccination? Since ~80% relative percent survival (rps) after 30 days of oral VP2-vaccination of both brown and rainbow trout was described before [11], the increase of the differential expression levels of some or all of the genes studied might be still required to obtain either higher rps and/or duration of the protection. Further

Table 2
Normalized values of selected genes in IPNV-infected and VP2-vaccinated rainbow trout 7 days after immunization by RTqPCR.

Gen	Organ	IPNV-infected	VP2-vaccinated
		Mean ± sd	Mean ± sd
stat1	Head Kidney	14.98 ± 0.44	*2.16 ± 0.01
	Spleen	1.33 ± 0.01	0.77 ± 0.12
	Intestine	3.75 ± 0.03	*0.71 ± 0.08
	Pyloric ceca	0.13 ± 0.00	0.36 ± 0.05
	Thymus	0.28 ± 0.00	*3.58 ± 0.03
ifn1	Head Kidney	30.17 ± 1.68	*9.99 ± 0.19
	Spleen	45.89 ± 0.17	*1.02 ± 0.16
	Intestine	103.97 ± 0.57	*2.16 ± 0.26
	Pyloric ceca	6.30 ± 0.02	*0.91 ± 0.13
	Thymus	11.20 ± 0.24	*45.25 ± 0.42
ifng	Head Kidney	74.29 ± 0.15	*1.66 ± 0.09
	Spleen	35.51 ± 0.38	*1.02 ± 0.16
	Intestine	100.80 ± 1.32	*0.51 ± 0.06
	Pyloric ceca	7.59 ± 0.02	*0.80 ± 0.11
	Thymus	9.92 ± 0.07	9.22 ± 0.06
mx1	Head Kidney	35.88 ± 0.36	*3.23 ± 0.02
	Spleen	2.41 ± 0.01	*0.33 ± 0.05
	Intestine	5.66 ± 0.03	*0.64 ± 0.07
	Pyloric ceca	0.75 ± 0.01	0.88 ± 0.12
	Thymus	0.56 ± 0.00	*2.79 ± 0.01
mx3	Head Kidney	0.67 ± 0.77	0.12 ± 0.15
	Spleen	0.20 ± 0.00	*0.01 ± 0.00
	Intestine	0.48 ± 0.00	*0.01 ± 0.00
	Pyloric ceca	0.79 ± 0.00	*0.13 ± 0.00
	Thymus	0.07 ± 0.00	0.15 ± 0.00
il8	Head Kidney	2304.12 ± 61.84	*117.38 ± 1.19
	Spleen	186.75 ± 0.79	*37.27 ± 5.83
	Intestine	621.67 ± 7.76	*44.94 ± 5.42
	Pyloric ceca	29.75 ± 0.10	33.13 ± 4.76
	Thymus	57.88 ± 1.07	*238.03 ± 2.90
il10	Head Kidney	7.39 ± 0.10	*0.33 ± 0.00
	Spleen	0.42 ± 0.00	*0.05 ± 0.00
	Intestine	1.40 ± 0.03	0.27 ± 0.03
	Pyloric ceca	0.05 ± 0.00	0.10 ± 0.01
	Thymus	0.15 ± 0.00	*0.57 ± 0.00
il11	Head Kidney	0.33 ± 0.01	0.03 ± 0.00
	Spleen	0.01 ± 0.00	0.00 ± 0.00
	Intestine	0.04 ± 0.00	0.01 ± 0.00
	Pyloric ceca	0.00 ± 0.00	0.01 ± 0.00
	Thymus	0.00 ± 0.00	0.01 ± 0.00
il12b	Head Kidney	15.30 ± 0.46	*1.82 ± 0.01
	Spleen	0.50 ± 0.00	0.50 ± 0.07
	Intestine	1.16 ± 0.07	0.56 ± 0.06
	Pyloric ceca	0.38 ± 0.00	0.41 ± 0.06
	Thymus	1.07 ± 0.02	*3.20 ± 0.04
tnf2	Head Kidney	2.02 ± 0.24	*0.18 ± 0.00
	Spleen	0.02 ± 0.00	0.02 ± 0.00
	Intestine	0.06 ± 0.00	0.03 ± 0.00
	Pyloric ceca	0.07 ± 0.00	0.02 ± 0.00
	Thymus	0.01 ± 0.00	0.11 ± 0.00
mhc1uda	Head Kidney	16.17 ± 0.15	*1.56 ± 0.11
	Spleen	2.50 ± 0.01	*0.53 ± 0.08
	Intestine	6.48 ± 0.04	*1.06 ± 0.12
	Pyloric ceca	0.45 ± 0.00	*0.18 ± 0.03
	Thymus	0.69 ± 0.00	*2.72 ± 0.01
igm	Head Kidney	236.39 ± 4.20	*6.08 ± 0.08
	Spleen	98.70 ± 0.57	*12.42 ± 1.94
	Intestine	228.33 ± 2.23	*2.44 ± 0.29
	Pyloric ceca	19.84 ± 0.07	*1.78 ± 0.25
	Thymus	21.04 ± 0.33	*7.94 ± 0.20
igt	Head Kidney	54.38 ± 1.47	*3.02 ± 0.02
	Spleen	0.72 ± 0.01	*0.05 ± 0.00
	Intestine	4.03 ± 0.07	*0.26 ± 0.00
	Pyloric ceca	0.35 ± 0.01	0.03 ± 0.00
	Thymus	0.21 ± 0.00	0.31 ± 0.00

The normalized values to *ef1a* were calculated by the $2^{-\Delta CT}$ method, according to the formula, $\Delta Ct = Ct$ gene-Ct_{ef1a}. 0.00 < 0.01.
*, significantly different when compared to their corresponding infected organ at the $p < 0.05$ level.

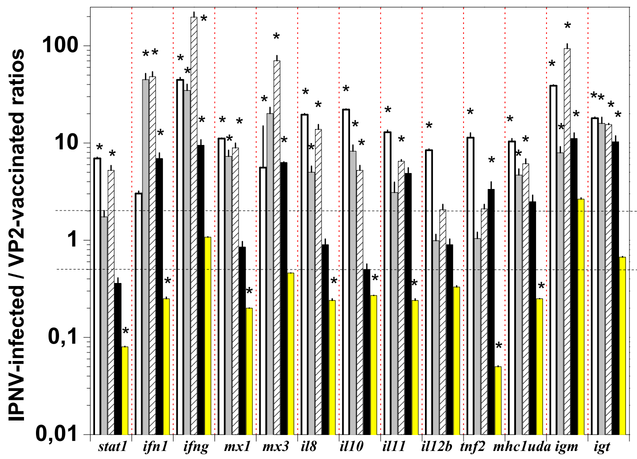

Fig. 4. Comparison of differential expression folds of selected genes in organs from IPNV-infected and VP2-vaccinated trout. Rainbow trout were infected with IPNV by 2 h immersion in 3 × 10^5 TCID/ml (mock infected with PBS) or orally vaccinated with pDNA-VP2 (mock vaccinated with pDNA) and their corresponding organs/tissues harvested 7 days later. The trout selected primers described in Table 1 were used to estimate differential transcript levels in different organs. Means of differential expression folds and their standard deviations were calculated for each of the organs from either IPNV-infected or VP2-vaccinated trout groups ($n = 3$, for each group) as indicated in methods. Ratios represented in the figure were calculated by the formula, folds from IPNV-infected trout/folds from VP2-vaccinated trout. Standard deviations (sd) were calculated as indicated in methods. Only the maximal sd were drawn for clarity. *, ratios significantly different from 2 or 0.5 at the $p < 0.05$ level. Horizontal dashed lines were drawn for ratios = 2 or 0.5. Red vertical dashed lines were drawn to separate genes. HK, open bars. SP, light grey bars. PC, hatched bars. IN, black bars. T, yellow bars.

work will clarify that possibility. It was also observed than a small but significant down-regulation occurred 2 days after oral vaccination in most of the studied genes (Fig. 3). It is most probable that the stress caused by the manipulation of trout during the artificial oral delivery method (a 20 μl tip introduced into the mouth–oesophagus of each trout) might have caused those effects.

Among the responses studied, several genes belonged to the IFN system. It is well known that the type I IFN system is involved in the first line of defense against viral infections and many reports have described its multiple functions in innate and adaptive immune responses. Their antiviral effect is acting through specific cell-surface receptors which trigger the JAK–STAT signal transduction pathway and a large number of genes known as IFN-stimulated genes (ISGs). Some of these genes encode antiviral proteins, such as Mx [40]. Thus, the salmon Mx protein has been shown to directly inhibit IPNV protein synthesis [29] or decrease viral infective titres

Table 3
Distribution of VP2 transcript relative values in organs from IPNV-infected and VP2-vaccinated rainbow trout.

Organ/tissue	IPNV-infected	VP2-vaccinated
	Mean relative value ± sd	Mean relative value ± sd
Head kidney	1961.46 ± 45.25	0.97 ± 0.08*
Spleen	80.32 ± 3.48	1.47 ± 0.03*
Intestine	865.69 ± 148.79	7.74 ± 0.33*
Pyloric ceca	47.27 ± 5.85	5.99 ± 0.32*
Thymus	24.30 ± 2.29	3.30 ± 0.04*

Rainbow trout were infected with IPNV by 2 h immersion in 3 × 10^5 $TCID_{50}$/ml. Relative values ($2^{-\Delta Ct}$) were multiplied by 10,000 to facilitate tabulation of the data. Means and standard deviations of VP2 relative values from different trout ($n = 3$) are represented.
*, significantly different when compared to their corresponding infected organ at the $p < 0.05$ level.

of either IPNV or IHNV [41]. In the present work, both *ifn1* and *ifng* were induced at high levels in SP, IN and PC in IPNV-infected trout while levels were also higher in T upon VP2-vaccination (Table 2). Furthermore, all the levels of IFN-related genes (*stat1*, *ifn1*, *ifng*, *mx1* and *mx3*) could be mimicked (ratios of IPNV-infected/VP2-vaccinated <1) in T by VP2-vaccination (Fig. 4), thus suggesting that the success of oral VP2-vaccination might be due to its capacity to induce such a response.

On the other hand, differential expression of IFNs and their induced genes have been reported in Atlantic salmon or trout [42,43] upon infection with IPNV. However, the virus could counteract those innate host defences as infection progressed. Thus, Skjesol et al. [44] reported on the ability of IPNV to redirect those initial cellular processes in favour of virus propagation while avoiding cellular antiviral responses and suggested VP4 and VP5 as candidate molecules to counteract the IFN response. These two proteins reduced the initial IFN-induced differential expression by acting on the *mx* promoter. The question might be relevant for the next generation of IPNV vaccines. Thus, in the light of our results with the oral VP2-vaccine, would it be better to avoid combination of VP2 with plasmids containing VP3 or VP4 to maintain an efficient immune defence system? Alternatively, would it be necessary to assay new vaccines with other immune genes looking for synergistic effects with the VP2?

Kinetic data reported after intraperitoneally injected IPNV showed an initial *stat1* up-regulation up to 4 days, as similarly estimated in this work (Fig. 3), which was later downregulated when the IPNV began to replicate [23]. High levels could be also induced in HK and T by VP2-vaccination when compared to IPNV-infection, suggesting that such an increase in this transcriptional regulator of the type I IFN system could be important to induce VP2-vaccine protection.

There is little information regarding pro- and anti-inflammatory cytokines on fish and their relation to viral infection and/or vaccination. It is known that recombinant trout *il8* attracts neutrophils [45] and it was induced by injected rhabdoviral DNA vaccines [30], while *il10* is a Th2-cytokine [25] suppressing excessive inflammatory responses [46] in response to *tnfa* [47]. Of all the genes examined in this work, *il8* showed the highest differential expression levels in all the organs, both in the IPNV-infected and the VP2-vaccinated trout (Table 2). Thus, up-regulated levels expressed as normalised relative values were from 57-fold in T to 2304-fold in HK in IPNV-infected trout, corresponding to 238-fold in T or 117-fold in HK in VP2-vaccinated trout (Table 2). In contrast, the anti-inflammatory *il10* had low differential expression levels in both the IPNV-infected (except in HK) and VP2-vaccinated trout. Reyes-Cerpa et al. [48] studying several interleukines during acute and persistent IPNV-infection of Atlantic salmon, found that the increased differential expression of *il10*, accompanied by the absence of induction of *il1b* and *il8*, indicates that IPNV triggers an anti-inflammatory response that may be part of the mechanisms to establish their persistence. According to our results, the levels of *il8* and *il10* in IPNV-infected fish were mimicked in VP2-vaccinated trout, although at lower levels and therefore in either case persistence seems to be avoided. Further studies are required to elucidate if up-regulation of *il8* and down regulation of *il10* could inhibit IPNV persistence and thus inhibit the establishment of an IPNV carrier state. This information would be also valuable for improvement of future vaccines against IPNV.

Bartee et al. [49] reported that synergy between *tnf* and *ifn* occurs mainly at the level of gene transcription, suggesting they could activate signal transduction pathways and transcription factors. *Tnf2* is an important pro-inflammatory cytokine, similar to *tnfa* [34] which is induced after viral infection [50], but not by killed virus or isolated viral proteins unless *ifng* is also provided [51]. If important for protection, however only in SP and T the *tnf2* levels obtained upon IPNV-infection could be mimicked by VP2-vaccination, perhaps showing a point in which oral vaccination could be improved.

Increased *il11* and *il12b* transcripts were found in the HK upon IPNV-infection. *Il12b* is one of the heterodimer chains of a Th1-cytokine also known in mammals as natural killer cell stimulatory factor 2 produced in macrophages, monocytes, dendritic cells and B lymphocytes in response to intracellular pathogens [52]. *Il12* has been used as vaccine adjuvant to increase protective mucosal immunity [53,54], but it has not been characterized nor tested in fish. Only in T could the transcript levels of *il11*/*il12b* be induced by the VP2-vaccine to higher fold levels than in IPNV-infection and therefore these interleukines might deserve further studies.

MHC-I molecules are involved in cell-mediated immunity by participating in antigen processing and presentation after viral infection and they are important for host antiviral immunity by regulating natural killer cell activity [55]. *Mhc1* was up-regulated after intramuscular injection of DNA vaccines to IPNV [56] and also in T after oral VP2-vaccination (Fig. 2C′). The levels of both *igm* and *igt* after IPNV-infection were 100–300-fold higher than the levels induced by the VP2-vaccine in HK (Fig. 3). However it is interesting to point out that *igm* and *igt* showed high levels of differential expression in IN and T after 7 days of vaccination (Fig. 2). The discovery of *igt* in trout [57] has changed the paradigm that *igm* was the only immunoglobulin class responding to antigenic challenge both in systemic and mucosal compartments. More recent work reveals that rainbow trout *igt* is an immunoglobulin specialized in mucosal immune responses [37]. With respect to the DNA vaccines many intriguing questions remain to be answered such as the immunoglobulin repertoires in systemic and mucosal sites of naïve and vaccinated fish or how *igt* and *igm* induction may be modulated by oral or intramuscular delivery. These deeper studies on mucosal responses are being designed for future work. In the rest of the organs similar levels of *igm*/*igt* were obtained (Fig. 4). Although IPNV neutralizing antibodies were demonstrated in the sera of VP2-vaccinated trout [11], whether those transcript levels correspond to specific antibodies or not will have to be demonstrated.

Differential expression of VP2 transcripts induced by VP2-vaccination was high and mimicked those caused by IPNV-infection even thought their levels of differential expression were lower. Thus, there is still room for improvement of oral VP2-vaccines by investigating ways to increase the present VP2 expression levels. Perhaps such an improvement could raise the efficacy of the VP2-vaccine in terms of both survival (>80%) and duration (>30 days). In this regard, we have defined some of the gene markers (i.e.: *ifn1*, *mx1*, *mx3*, *tnf2*, *igm*, *igt*) whose expression could be followed during future oral vaccine formulation development (DNA-vaccine dose, inclusion of other DNA delivery agents, synergy with plasmid adjuvants, etc).

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1.3. Modulación de genes relacionados con el reclutamiento de células inmunes en el tracto digestivo de truchas infectadas con IPNV o truchas vacunadas por vía oral con pcDNA-VP2

En este trabajo se estudió la expresión de quimioquinas y de receptores de quimioquinas en los cinco segmentos del intestino de peces vacunados con pcDNA-VP2 por vía oral comparándolos con la expresión inducida por el virus IPN. Las quimioquinas estudiadas fueron: CK9, CK10, CK11, CK12, CCR7, CCR9 y CCR9B; además del complejo mayor de histocompatibilidad (MHC-II) y el factor de necrosis tumoral α (TNF2α) como marcadores de la presentación antigénica e inflamación, respectivamente.

Con técnicas inmunohistoquímicas se analizó la presencia de células IgM^+, IgT^+, CD3 (marcador de linfocitos T) a lo largo del tracto digestivo

Diseño experimental:

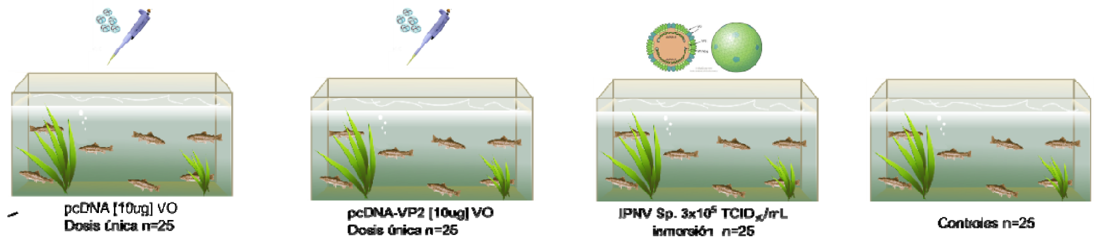

Figura 1: Diseño experimental

Alevines de trucha arco iris de aproximadamente 2 g, 4–5 cm de longitud

- Grupo de peces (n=30) vacunados con 10 µg de pcDNA-VP2 por vía oral “vacunados”.
- Grupo de peces (n=30) vacunados con 10 µg de pcDNA por vía oral “plásmido vacío”.
- Grupo de peces (n=30) infectados por inmersión con 3 $x10^5$ $TCID_{50}$/ml de IPNV Sp “Infectados”
- Grupo de peces (n=30) sin tratamiento “Control”

Tiempos de muestreos: 1, 3 y 7 días post-tratamiento. 6 individuos por grupo.

Muestras: Intestino dividido en cinco segmentos: (esófago, estómago, intestino ciego pilórico, medio y posterior) n=6. Ver figura 2.

Métodos: Extracción de RNA total utilizando TriZol, síntesis de cDNA, PCR cuantitativa a tiempo real y Análisis de inmunohistoquímica.

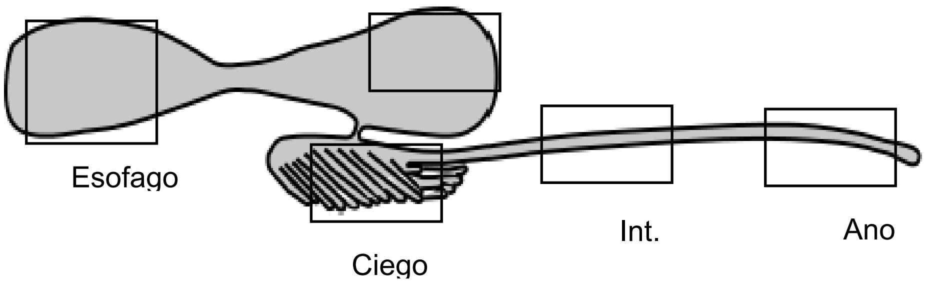

Figura 2: Representación esquemática de los segmentos del tracto digestivo de la trucha arco iris

Resumen:

Cuando comparamos la respuesta inmune del intestino (5 segmentos) de los peces infectados con IPNV con la respuesta a la vacunación oral con pcDNA-VP2 observamos que la transcripción de las quimioquinas, receptores de quimioquinas, MHC y TNF se expresan en todos los segmentos del intestino con diferentes niveles de expresión. En este estudio, se identificaron por primera vez linfocitos T CD3^{+} en el intestino anterior, ciego pilórico, intestino medio y posterior de los peces infectados con IPNV.

En resumen, en trucha arco iris la infección con virus IPN por inmersión modula la transcripción de varias quimioquinas como CK9, CK10, CK11, CK12, CCR7, CCR9, CCR9B, MHC-II y TNFγ, al igual que moviliza células IgM^{+}, IgT^{+} y linfocitos T CD3^{+} en diferentes segmentos del tracto intestinal. Además, las células epiteliales ubicadas en los diferentes segmentos del intestino, responsables de la producción de quimioquinas son estimuladas de diferentes maneras a lo largo del tracto intestinal, ya sea por el virus IPN como por la vacuna pcDNA-VP2.

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Modulation of genes related to the recruitment of immune cells in the digestive tract of trout experimentally infected with infectious pancreatic necrosis virus (IPNV) or orally vaccinated

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ABSTRACT

There are still many details of how intestinal immunity is regulated that remain unsolved in teleost. Although leukocytes are present all along the digestive tract, most immunological studies have focused on the posterior segments and the importance of each gut segment in terms of immunity has barely been addressed. In the current work, we have studied the regulation of several immune genes along five segments of the rainbow trout (*Oncorhynchus mykiss*) digestive tract, comparing the effects observed in response to an infectious pancreatic necrosis virus (IPNV) infection to those elicited by oral vaccination with a plasmid coding for viral VP2. We have focused on the regulation of several mucosal chemokines, chemokine receptors, the major histocompatibility complex II (MHC-II) and tumor necrosis factor α (TNF-α). Furthermore, the recruitment of IgM⁺ cells and CD3⁺ cells was evaluated along the different segments in response to IPNV by immunohistochemical techniques. Our results provide evidences that there is a differential regulation of these immune genes in response to both stimuli along the gut segments. Along with this chemokine and chemokine receptor induction, IPNV provoked a mobilization of IgM⁺ and IgT⁺ cells to the foregut and pyloric caeca region, and CD3⁺ cells to the pyloric caeca and midgut/hindgut regions. Our results will contribute to a better understanding of how mucosal immunity is orchestrated in the different gut segments of teleost.

1. Introduction

Although the structures and segments present in the digestive tract show significant differences among the diverse teleost species, a general division into three main segments has been established (Rombout et al., 2011). The first segment or foregut is where the food protein uptake takes place, with enterocytes acting as absorptive cells. This segment includes the esophagus and in some species, a defined stomach. The second segment is characterized by a strong uptake of macromolecules and enterocytes containing large supranuclear vacuoles. This segment includes the midgut and in some species such as salmonids, a variable number of pyloric caeca (pyloric appendages) near the pylorus. Fish caeca are an adaptation to increase gut surface area, contributing to a higher macromolecule uptake than that of the rest of the digestive tract. Finally, the third segment is the hindgut in which enterocytes are thought to have an osmorregulatory function, and includes an anal region that in certain species can constitute a proper rectum separated by valves.

Most previous studies related to immunological properties of the teleost digestive tract have focused on the last segments (Rombout et al., 2011), and very poor attention has been given to the other segments from an immunological point of view. In rainbow trout (*Oncorhynchus mykiss*), we have defined in a previous study that IgM⁺ and IgT⁺ cells are present all along the digestive tract, with the exception of the stomach (Ballesteros et al., 2013). IgM⁺ cells were mostly located in the lamina propria (LP) and as intraepithelial lymphocytes (IELs) in the pyloric caeca region. On the other hand, IgT⁺ cells were primarily localized as IELs. Furthermore, when fish were orally vaccinated with an alginate-coated DNA vaccine against infectious pancreatic necrosis virus (IPNV), a significant recruitment of B lymphocytes to the pyloric caeca region was observed. Furthermore, significant differences in the transcription of Ig genes and B cell transcription factors were only observed between vaccinated and control fish in this segment (Ballesteros et al., 2013). Hence, in this study, we wanted to compare the effects of oral DNA vaccination in the different gut

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segments to those elicited by a bath infection with IPNV. In this occasion, we have focused on the regulation of several chemokines and chemokine receptor genes that are related to mucosal responses, namely CK9, CK10, CK11, CK12, CCR7, CCR9 and CCR9B. We have also included the evaluation of transcription of the major histocompatibility complex (MHC-II) and tumor necrosis factor α (TNF-α) as indicators of antigen presentation and inflammation respectively. CK9, CK10, CK11 and CK12 chemokines are strongly expressed in rainbow trout mucosal tissues and are regulated in gills and skin in response to viral infections (Montero et al., 2011). On the other hand, the chemokine receptor CCR7 plays an important role in the recruitment of different leukocyte subtypes such as dendritic cells, T and B lymphocytes to the intestine in mammals (Jang et al., 2006; Okada et al., 2002; Wurbel et al., 2007). CCR7 has been recently identified in rainbow trout, where it also appears to be implicated in mucosal immunity (Ordas et al., 2012). In this species, CCR7 transcription was up-regulated in gut IgM$^+$ and IgT$^+$ cells from fish infected with an intestinal parasite in comparison to mock-infected controls (Ordas et al., 2012). Mammalian CCR9, in combination with other chemokine receptors, mediates the homing of antigen-secreting IgA$^+$ cells to the mucosal LP (Hieshima et al., 2004). In rainbow trout, two CCR9 genes, designated as CCR9 (Daniels et al., 1999) and CCR9B (Dixon et al., 2013) were identified and are known to be differentially regulated (Dixon et al., 2013). Along with this evaluation of immune genes related to leukocyte mobilization, we have also studied through immunohistochemical techniques the actual recruitment of different leukocyte types to the different gut segments in response to the IPNV infection. We have analyzed the presence of IgM$^+$ and IgT$^+$ cells, as well as cells expressing CD3, a marker for T lymphocytes along the digestive tract in control and infected fish. As previously described by (Bernard et al. 2006), these CD3$^+$ cells were located as IELs, however, our results provide the first evidence in teleost of a mobilization T IELs cell to the digestive tract in response to a viral infection.

2. Materials and methods

2.1. Fish

Healthy specimens of rainbow trout (*O. mykiss*) of approximately 4–5 cm (approximately 2 g) were obtained from a local spring water farm with no history of viral disease. Fish were maintained at the Centro de Investigaciones Biológicas (CIB-CSIC) laboratory at 15 °C with a re-circulating water system, 12:12 h light: dark photoperiod and fed twice a day with a commercial diet (Skretting, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed. In addition, two pools of five fish were tested by standard methods to confirm the absence of any salmonid virus by isolation using BF cells (Alonso et al., 1999). The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were previously approved by the local Ethics committee.

2.2. IPNV DNA vaccine

The DNA vaccine (pcDNA-VP2) was prepared as described previously (de Las Heras et al., 2008), by inserting the IPNV-VP2 gene into the pcDNA.3.1/V5/His-TOPO expression vector (Invitrogen, USA). The empty re-ligated plasmid was used as a control (pcDNA). The pcDNA-VP2 and pcDNA plasmids were coated with alginate, preparing the microspheres as previously described (de las Heras et al. 2010).

2.3. Virus propagation

The IPNV Sp strain obtained from the ATCC (ATCC VR 1318) was propagated in the BF-2 cell line from bluegill fry (*Lepomis macrochirus*, ATCC-CCL 91) with Leibovitz's medium (L15, Gibco, Spain) supplemented with 100 IU/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine and 2% fetal bovine serum (FBS, Gibco, Spain) at 20 °C. Supernatants from IPNV infected BF-2 cell monolayers were clarified by centrifugation at 1000*g* for 20 min after cytophatic effect was extensive. Clarified supernatants were used for the experiments. Viral titration was performed in 96 well culture plates. The infective titers were determined as the 50% infective dose in tissue culture ($TCID_{50}$/ml) according to the method described by Reed and Muench (Reed and Muench 1938).

2.4. Experimental design

In order to compare the mucosal immune regulation elicited by IPNV infection with that provoked by oral DNA IPNV vaccination in the different segments of the digestive tract, rainbow trout were either orally vaccinated with alginate-coated pcDNA-VP2 or infected with IPNV by immersion in parallel experiments using corresponding control groups.

For the immunization, trout were divided into two different groups. One group was orally vaccinated with 10 μl of the vaccine microsphere suspension containing 10 μg of pcDNA-VP2, while a second group received 10 μg of the pcDNA empty plasmid diluted in 10 μl of a microsphere suspension. Vaccination was performed with an automatic pipette with a 20 μl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the digestive tract. For the IPNV bath infection, rainbow trout were transferred to 2 l of a viral solution containing IPNV Sp strain (5×10^5 $TCID_{50}$/ml). After 1 h of viral adsorption with strong aeration at 15 °C, each experimental group was transferred to an individual water tank. Mock-infected groups were also transferred to 2 l tanks containing an equivalent amount of non-infected culture media. After 1 h of strong aeration, they were also moved to their corresponding tanks. The water-quality parameters were maintained at optimal levels and equal in all tanks.

In a preliminary experiment using this set-up, four fish infected with IPNV and four fish vaccinated with pcDNA-VP2 were euthanized with an overdose of MS-222 at days 1, 3 and 7 post-treatment and the esophagus, stomach, pyloric caeca, midgut and hindgut removed as described before (Ballesteros et al., 2013) and placed in Trizol reagent (Invitrogen) for posterior RNA extraction. In this preliminary experiment, unhandled fish sampled at day 0 were used as controls. This experiment was used to determine that 7 days was the time point at which most chemokine genes were modulated in response to the different stimuli. Thus, the experiment was repeated once more sampling six fish from each of the experimental groups previously described at day 7 post-stimulation. In the case of the IPNV-infected and the mock-infected control groups, four additional trout were sampled for immunohistochemistry.

2.5. Gene expression analysis

Total RNA was isolated from these tissues using Trizol® reagent (Invitrogen) according to manufacturer's instructions. Five microgram of RNA were used to obtain cDNA in each sample using the Super Script™ II kit (Invitrogen) and oligo $(dT)_{12-18}$ (25 pmol/μl) following manufacturer's instructions. The resulting cDNA was diluted and stored at −20 °C.

Real-time PCR amplification was carried out in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Spain). The analysis of the immune genes was performed using Quantimix Easy Master

Mix (Biotools, Spain) as described previously (Ballesteros et al., 2012a,b). All the primers used are shown in Table 1 and had already been optimized in previous studies (Dixon et al., 2013; Montero et al., 2011; Zou et al., 2002). The thermal profile used was 10 min at 95 °C, followed by 40 amplification cycles (30 s at 95 °C and 1 min at 60 °C) and a dissociation cycle (30 s at 95 °C, 1 min 60 °C and 30 s at 95 °C). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. Elongation factor 1α (EF-1α) was used as house-keeping control gene. The expression of target genes was calculated as the relative values ($2^{-\Delta CT}$) or fold change relative to EF-1α expression according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1
Oligonucleotides and probes used for real time PCR in this study.

Gene	Primer	Sequence (5′-3′)
EF-1α	rtEF1α F	GATCCAGAAGGAGGTCACCA
	rtEF1α R	TTACGTTCGACCTTCCATCC
CK9	rtCK9 F	GGCTCTTATGGGAACTGCTG
	rtCK9 R	CTGGGATTGGCACAAACAG
CK10	rtCK10 F	ATTGCCAAGATCCTCTTCTGTGTTC
	rtCK10 R	CCTGAGGCTGGTAACCTATGACAAC
CK11	rtCK11 F	CCTTTGAGCATACTAATGCGAGTGG
	rtCK11 R	GTCTGCACAATACTTCCTCCCATTG
CK12	rtCK12 F	GACATCGATGCCACTGTGTT
	rtCK12 R	GGAGATGGTTCGCTCCAGAC
CCR7	CCR7 4F	TTCACTGATTACCCCACAGACAATA
	CCR7 4R	AAGCAGATGAGGGAGTAAAAGGTG
CCR9	CCR9 F	TCAATCCCTTCCTGTATGTGTTTGT
	CCR9 R	GTCCGTGTCTGACATAACTGAGGAG
CCR9B	CCR9B F	AATATTTCCAACGTCTGAAACAGGA
	CCR9B R	CTCACCCAGGACTTATCACACATTC
MHC-IIα	rtMHCIIα F	ACACCCTTATCTGCCACGTC
	rtMHCIIα R	TCTGGGGTGAAGCTCAGACT
TNF-α	TNF2 Fw	TGCTGCTCCATGTGTGGTGC
	TNF2 Rev	AGGGACGGGGAGCCTTGAT
VP2	VP2 F	GCCAAGATGACCGAGTCCAT
	VP2 R	TGACAGCTTGACCCTGGTGAT
	VP2 probe	CCGACCGAGAACAT
β-actin	Actin-F	GGCCGTGTTGTCCCTGTAC
	Actin-R	CCTCTGGCCGTACCACC
	Actin-probe	CCGGAGTCCATGACGATACC

To analyze the transcription of the viral VP2 genes along the different gut segments, a Taq-Man probe and primers designed to the VP2 gene were used as described before (Rodriguez Saint-Jean et al., 2010). The probes were dual labeled with a reporter dye at the 5′ end (FAM, 6-carboxy fluorescein) and a quencher dye at the 3′ end (TAMRA, 6-carboxytetramthyrhodamine). In this case, β-actin was used as a reference house-keeping gene. Primers and probes were synthesized at Applied Biosystems and are also shown in Table 1. PCR amplification was performed in a final volume of 20 μl by adding 1 μl of cDNA, 10 μl of iQ supermix (Bio Rad), 8 μl of dH2O and 1 μl of a 20 × mix containing the forward primer (18 μM), reverse primer (18 μM) and probe (5 μM). The cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence output of each cycle was measured and recorded upon completion of the entire run, and a relative quantification of the transcripts was performed.

The data obtained were analyzed using the iQ5 optical system software version 2.0 (BioRad). All PCR reactions were performed in duplicate, expressing the results as the mean ± standard deviation.

2.6. Immunohistochemistry

Segments from the digestive tract obtained from control and IPNV-infected fish were fixed in Bouin's solution for 24 h, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 μm. After dewaxing and rehydration, some sections were stained with hematoxylin–eosin in order to determine the levels of infiltration, apparent damages or pathological changes. A second set of sections was subjected to an indirect immunocytochemical method for detection of trout IgM, IgT and CD3. The anti-IgM and anti-IgT monoclonal antibodies were kindly donated by Dr. Kurt Buchmann from the University of Copenhagen and Dr. Karsten Skjoedt from the University of Southern Denmark (Denmark) (Olsen et al., 2011; von Gersdorff Jorgensen et al., 2011). These antibodies recognize both the membrane and the secreted forms of the Igs. The anti-CD3 antibody was kindly provided by Dr. Erin Bromage from the University of Massachusetts Dartmouth (USA) (Boardman et al., 2012). Endogenous peroxidase was inhibited after rehydration of the sections by 10 min incubation in 3% H_2O_2 in PBS. After a heat induced epitope retrieval in Tris–EDTA buffer pH 9.0 (800 w for 5 min and 450 w

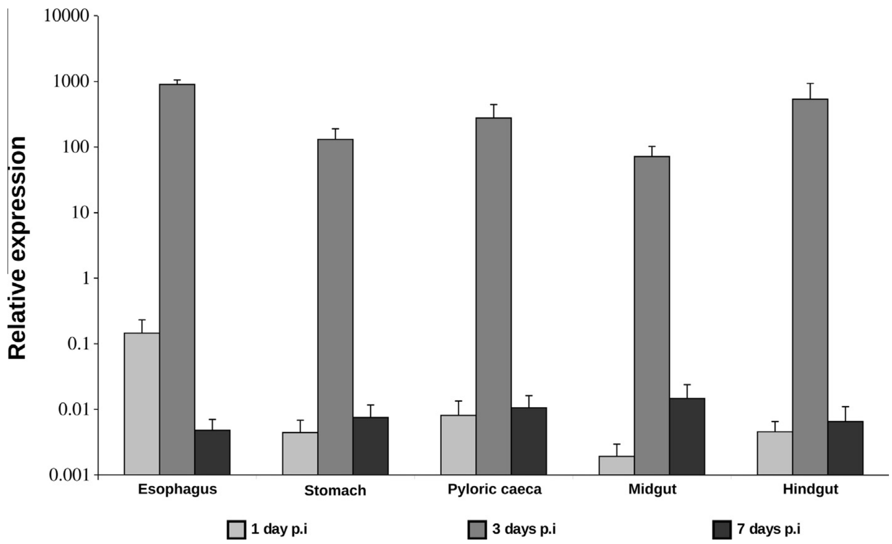

Fig. 1. VP2 transcription in the different segments of the digestive tract of IPNV infected fish. Fish were infected through immersion with IPNV Sp strain (5×10^5 $TCID_{50}$/ml) and sampled at days 1, 3 and 7 post-infection. Data are shown as relative transcription levels of VP2 normalized to the transcription of the house-keeping gene β-actin at different days post-infection ± SD ($n = 4$).

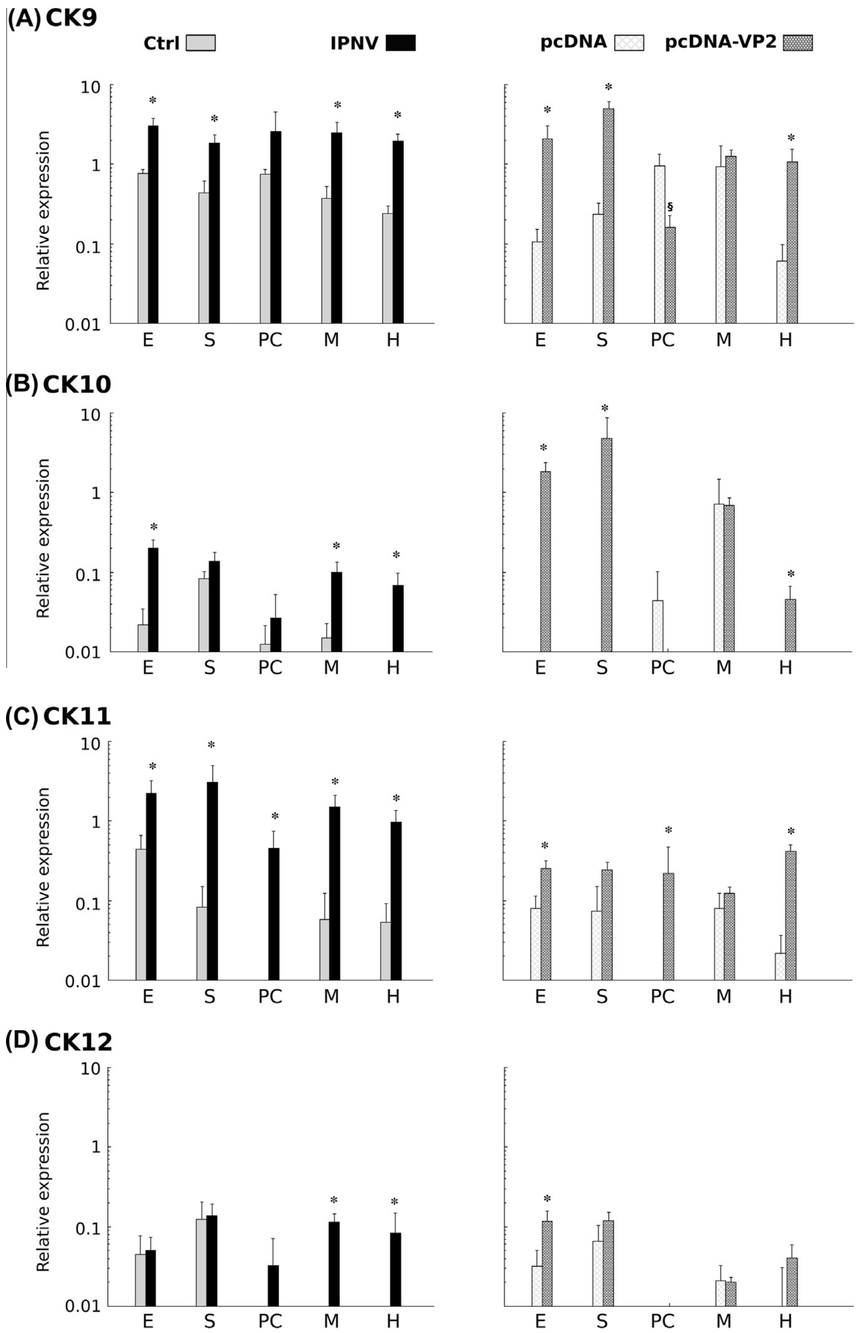

Fig. 2. Mucosal chemokine modulation in response to IPNV infection or oral DNA vaccination. Left side: Trout were infected with IPNV Sp strain (5×10^5 $TCID_{50}$/ml) or mock-infected in the same conditions. Right side: Trout were orally vaccinated with 10 μl of suspension of the vaccine microspheres containing either 10 μg of pcDNA-VP2 or 10 μg of the pcDNA empty plasmid diluted in PBS. In all cases, trout were sacrificed after 7 days and the different segments of the digestive tract removed for RNA extraction and analysis of immune gene transcription through real time PCR. E: esophagus; S: stomach; PC: pyloric caeca; M: midgut; H: hindgut. Levels of CK9 (A), CK10 (B), CK11 (C) and CK12 (D) transcription in the different segments were studied through real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α ± SD ($n = 6$). The relative significance of differences between treatment groups and their respective controls at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as ∗ (up-regulations) or § (down-modulations).

for 5 min in a microwave oven), the sections were pre-incubated in two different blocking solutions consisting of 2% BSA (bovine serum albumin; Sigma–Aldrich) in TBT (Tris buffer with 0.02% tween 20) at room temperature for 30 min, and 10% normal goat serum in TBT for 30 min. Then, sections were incubated with primary antibody solution overnight at 4 °C. Monoclonal mouse anti-trout IgM was used in a dilution of 1:150 while monoclonal mouse anti-trout IgT and anti-trout CD3 were added at a 1:300 dilution. Following this incubation, unbound primary antibodies were washed off using TBT. The tissue was covered with anti-mouse EnVision™ System HRP labelled secondary antibody (Dako) and left for a 30 min incubation period at room temperature. Subsequently, the tissue was washed three times with TBT and then incubated in AEC substrate [0.05 M acetic acid buffer (pH 5) with 0.015% H_2O_2 and 0.4 g/l 3-amino-9-ethylcarbazole (Alfa Aesar)] for 15 min and afterwards washed for 4 min in tap water. The specificity of the reactions was determined by omitting the primary antibodies. Mayer's haematoxylin (Dako) was used as nuclear counter stain, and mounting was conducted

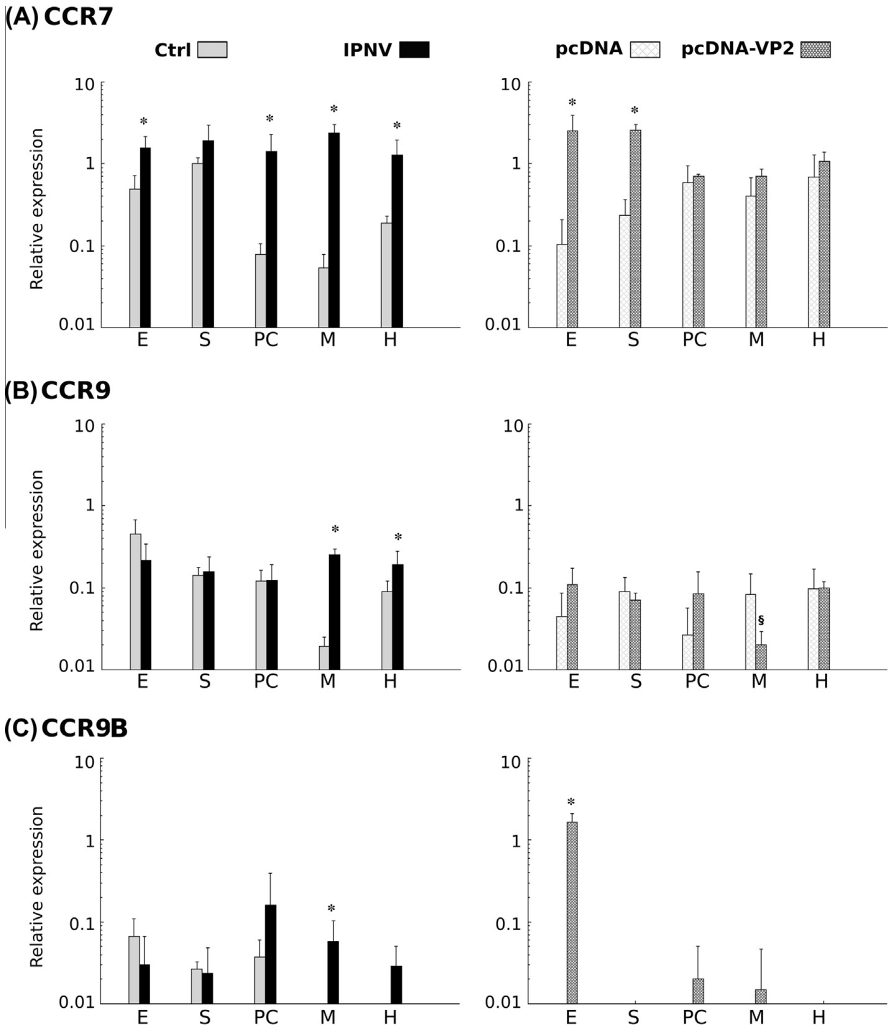

Fig. 3. Mucosal chemokine receptor modulation in response to IPNV infection or oral DNA vaccination. The experimental design is described in the legend of Fig. 2. Levels of CCR7 (A), CCR9 (B), and CCR9B (C) transcription in the different segments were studied through real time PCR. E: esophagus; S: stomach; PC: pyloric caeca; M: midgut; H: hindgut. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α ± SD ($n = 6$). The relative significance of differences between treatment groups and their respective controls at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as ∗ (up-regulations) or § (down-modulations).

with Aquamount (Merck). Slides were examined with an Axiolab (Zeiss) light microscope.

2.7. Statistical analysis

Prior to statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro–Wilk test. Analysis of variance (factorial ANOVA) were run to determine whether the differentially expressed gene differed between the replicates for an individual gene, followed by Tukey's multiple comparison test for differences between experimental groups. The Student's *t* test was used also to compare some paired samples. All statistics were run in SPSS Version 15. *P* values less than 0.05 were considered significant.

3. Results

3.1. IPNV replication along the digestive tract

In a previous study, we had established that after oral vaccination with an IPNV DNA vaccine, VP2 transcription could be detected all along the trout digestive tract (Ballesteros et al., 2013). To verify, whether IPNV is also capable of replicating in all the different gut segments after a bath infection, we studied VP2 transcription through real time PCR in the samples obtained from the IPNV-infected group. VP2 transcription was detected along all the gut segments from day 1 to day 7 post-infection (Fig. 1). At day 1 post-infection, the VP2 mRNA levels were higher in the esophagus in comparison to the other segments. The levels of VP2 transcription peaked at day 3, to decrease again at day 7. At both of these time points, however, no differences were observed in VP2 mRNA levels among the different segments.

3.2. Modulation of mucosal chemokines in the different gut segments

The main aim of our study was to compare the immune response elicited by IPNV bath infection in the different gut segments to that observed in response to oral DNA vaccination. As a preliminary step, we performed an experiment, in which four fish per group were sampled at different time points in the IPNV-infected and the pcDNA-VP2-vaccinated groups and CK9, CK10, CK11, CK12 and CCR7 mRNA levels studied in comparison to the levels

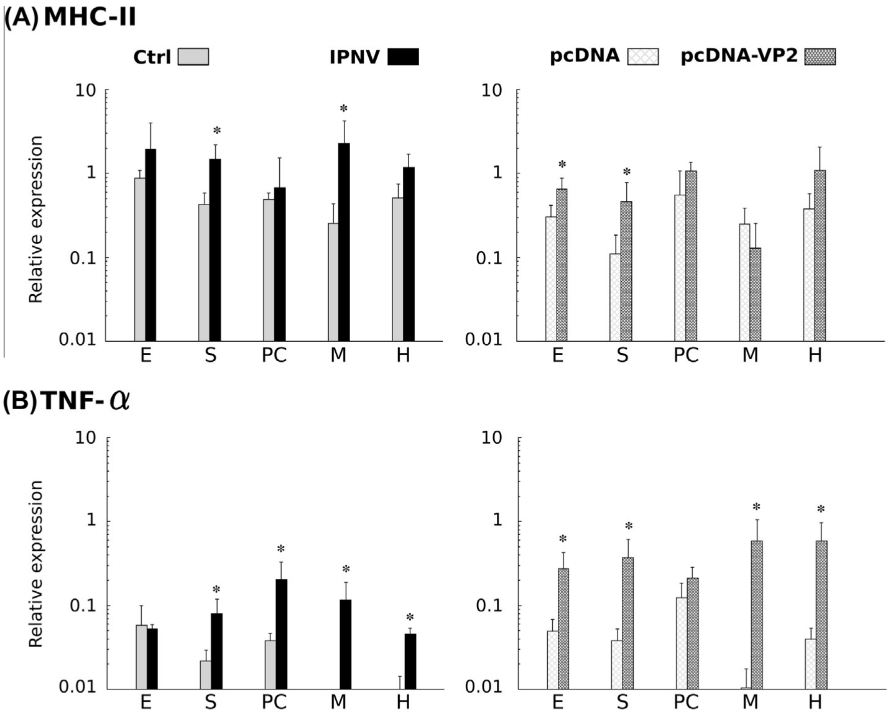

Fig. 4. MHC-II and TNF-α modulation in response to IPNV infection or oral DNA vaccination. The experimental design is described in the legend of Fig. 2. Levels of MHC-II (A) and TNF-α (B) transcription in the different segments were studied through real time PCR. E: esophagus; S: stomach; PC: pyloric caeca; M: midgut; H: hindgut. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α ± SD ($n = 6$). The relative significance of differences between treatment groups and their respective controls at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as ∗.

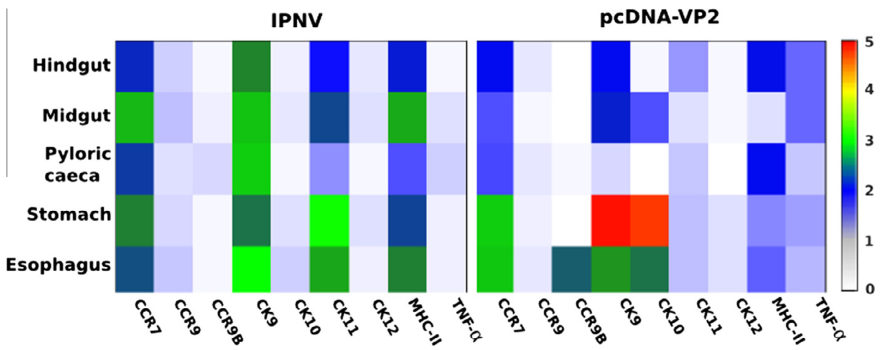

Fig. 5. Heat map depicting relative expression profiles of regulated genes in the segments of the digestive tract from rainbow trout. Fish were infected with IPNV or vaccinated with the pcDNA-VP2 plasmid and processed after 7 days. Levels of relative expression are represented by a range of colours from the lower (grey) to the higher (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed in unhandled fish before treatment. Once the transcription of these genes was determined in the different segments (Supplementary Fig. 1), we established that 7 days post-stimulation was the adequate time point to study these responses in both the infected and the vaccinated groups, because it was at this time point when most of the significant gene modulations were observed in all segments. Therefore, the experiment was repeated and six fish per group were sampled in all experimental groups at day 7 post-stimulation exclusively.

Firstly, we studied the transcription of CK9, CK10, CK11 and CK12 chemokines. The essential role that these four chemokines play in mucosal responses (gills and skin) has been previously demonstrated using a VHSV bath infection model (Montero et al., 2011). In the current work, in response to IPNV, CK9 transcription was up-regulated in all gut segments, although differences were only significant in esophagus, stomach, midgut and hindgut (Fig. 2A). The VP2 vaccine also produced an up-regulation of CK9 transcription in the esophagus, the stomach and hindgut, but not in the midgut (Fig. 2A). On the other hand, pcDNA-VP2 significantly down-modulated CK9 transcription in the pyloric caeca (Fig. 2A). In the case of CK10, IPNV significantly up-regulated the chemokine mRNA levels in the esophagus, midgut and hindgut, whereas the oral vaccine up-regulated its transcription in the esophagus, the stomach and hindgut (Fig. 2B). CK11 transcription significantly increased in all gut segments in response to IPNV (Fig. 2C), but only in the esophagus, pyloric caeca and hindgut of vaccinated fish (Fig. 2C). Finally, CK12 mRNA levels increased only in the midgut and hindgut after IPNV infection (Fig. 2D), and only in the esophagus after oral IPNV vaccination (Fig. 2D).

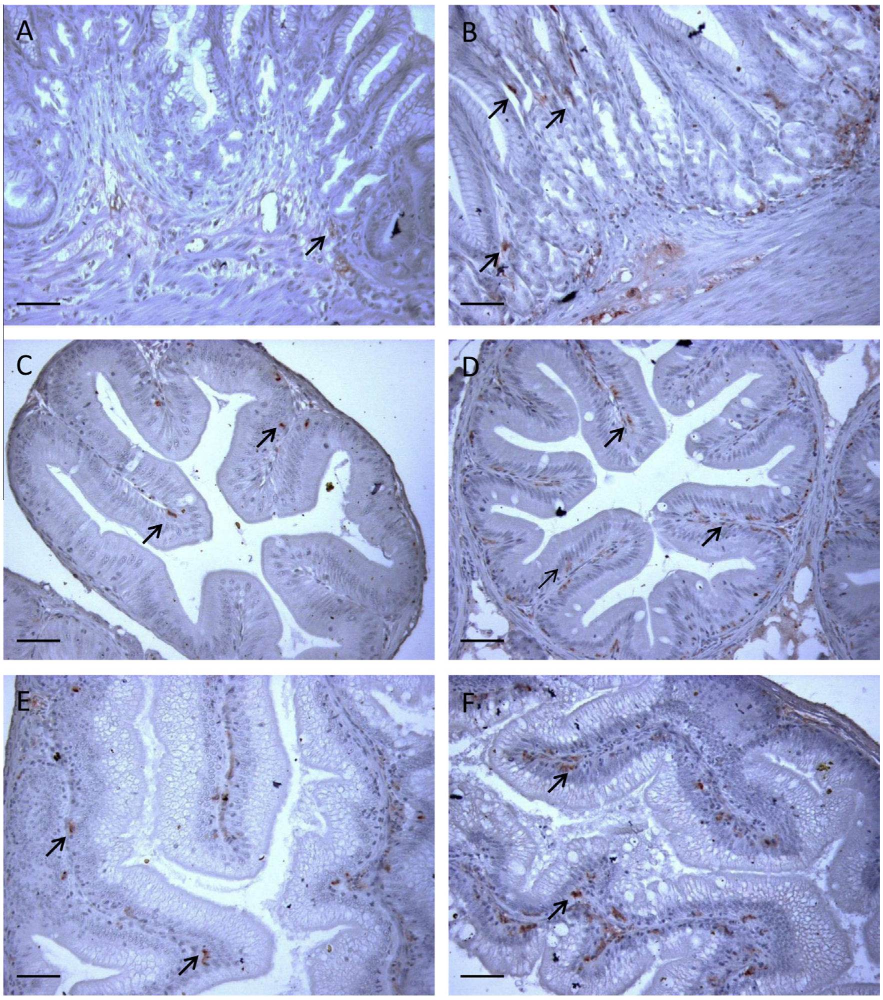

Fig. 6. IgM+ cell detection in the different gut segments in IPNV-infected fish and mock-infected fish. Immunohistochemical detection of trout IgM+ cells in the foregut (A and B), pyloric caeca (C and D) or midgut/hindgut region (E and F) of mock-infected fish (A, C, E) or IPNV infected fish (B, D, F). Trout were infected with IPNV as described in the legend of Fig. 1 and sampled at day 7 post-infection. Arrows indicate examples of positive cells. Bar: 200 µm.

3.3. Modulation of chemokine receptors in the different gut segments

We also studied the transcription of CCR7, CCR9 and CCR9B chemokine receptors, since these receptors are implicated in the recruitment of immune cells to the intestine in mammals (Okada et al., 2002; Wurbel et al., 2007). IPNV significantly increased CCR7 mRNA levels in all the gut segments except the stomach (Fig. 3A), whereas the pcDNA-VP2 vaccine only increased CCR7 transcription in the esophagus and the stomach (Fig. 3A). CCR9, on the other hand, was only significantly up-regulated in midgut and hindgut of virus infected fish (Fig. 3B). In response to the oral DNA vaccine, no significant up-regulations of CCR9 levels were detected, but the vaccine significantly down-modulated CCR9 transcription in the midgut (Fig. 3B). Finally, IPNV significantly increased the transcription of CCR9B in the midgut (Fig. 3A), whereas pcDNA-VP2 significantly increased CCR9B transcription only in the esophagus.

3.4. Modulation of MHC-II and TNF-α1 in the different gut segments

To study whether this modulation of chemokines and chemokine receptors correlated with an increased antigen presentation or with a pro-inflammatory response, the levels of expression of MHC-II and TNF-α were also assayed. MHC-II mRNA levels were quite high in all gut segments, and were significantly up-regulated in response to IPNV in the stomach and midgut (Fig. 4A). When fish were orally vaccinated, MHC-II transcription was only up-regulated in the esophagus and the stomach (Fig. 4B). In the case of TNF-α, IPNV increased its mRNA levels in all gut segments with the exception of the esophagus (Fig. 4B). Orally-vaccinated fish, on the other hand, had increased TNF-α mRNA levels in all gut segments except the pyloric caeca (Fig. 4B). A summary of the fold increases observed in infected and vaccinated groups in comparison to their corresponding control groups is shown in Fig. 5 for these and all of the other genes studied in this work.

3.5. IgM+ cell mobilization in response to IPNV along the digestive tract

In a previous study, we had examined the mobilization of IgM+ cells to an oral DNA vaccination with pcDNA-VP2 (Ballesteros et al., 2013). That study revealed that IgM+ cells were constitutively present in all gut segments with the exception of the stomach and in response to the vaccine were recruited mainly in the pyloric caeca region. Thus, in the current study, we evaluated the presence of IgM+ cells along the digestive tract in fish infected with IPNV in comparison to mock-infected fish. In the foregut, most IgM+ cells were present in the LP (Fig. 6A), as previously described (Ballesteros et al., 2013). When fish were infected with IPNV, the

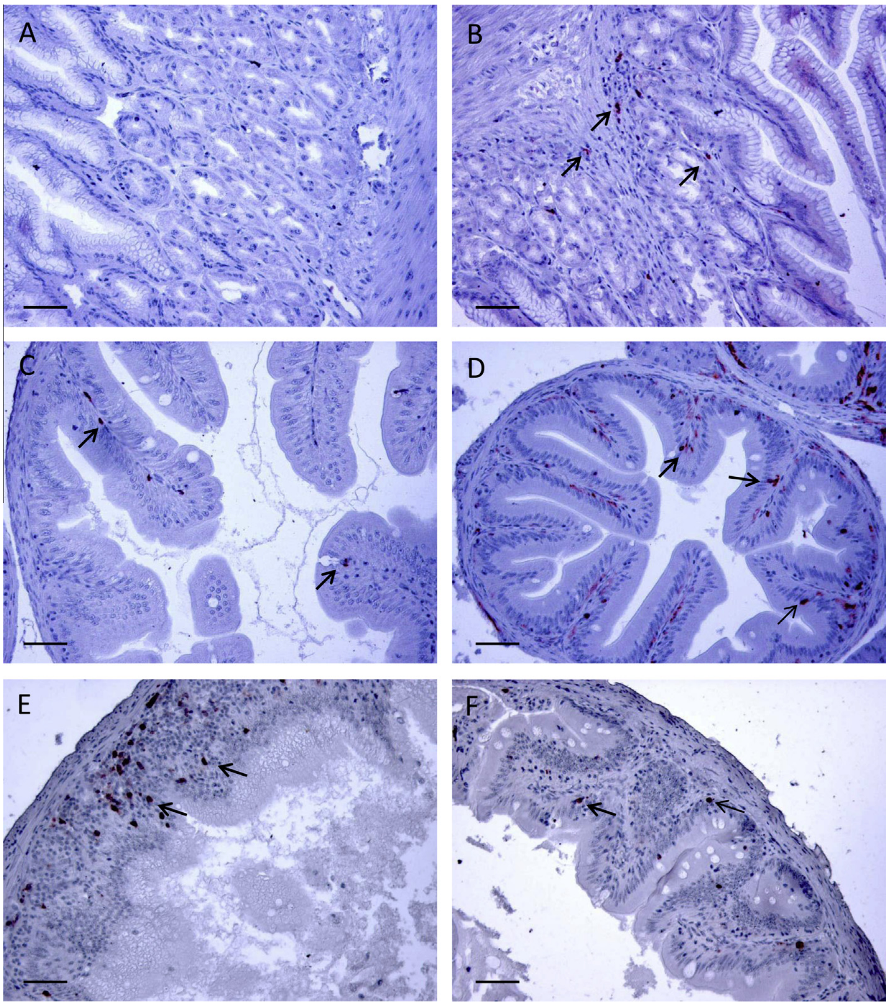

Fig. 7. IgT$^+$ cell detection in the different gut segments in IPNV-infected fish and mock-infected fish. Immunohistochemical detection of trout IgT$^+$ cells in the foregut (A and B), pyloric caeca (C and D) or midgut/hindgut region (E and F) of mock-infected fish (A, C, E) or IPNV infected fish (B, D, F). Trout were infected with IPNV as described in the legend of Fig. 1 and sampled at day 7 post-infection. Arrows indicate examples of positive cells. Bar: 200 μm.

number of IgM$^+$ in this area increased, while some reactivity was also observed in the apical surface of the enterocytes. In the pyloric caeca region, accordingly with what was published before (Ballesteros et al., 2013), IgM$^+$ cells were detected as IELs (Fig. 6C). In response to IPNV infection, the number of IgM$^+$ cells strongly increased in this area (Fig. 6D). Finally, in the hindgut/midgut area, IgM$^+$ cells were mostly localized in the LP (Fig. 6E), but in this case the number of cells was not consistently different in IPNV-infected fish (Fig. 6F).

3.6. IgT$^+$ cell mobilization in response to IPNV along the digestive tract

In the foregut, very few scattered IgT$^+$ cells were observed in some sections (Fig. 7A). When fish were exposed to IPNV, more IgT$^+$ cells could be detected (Fig. 7B) in the tissue samples, even though still very few positive cells were present in this segment. In the pyloric caeca region, many more IgT$^+$ cells were observed, mainly as IELs (Fig. 7C). As in the case of IgM$^+$ cells, IgT$^+$ cells were also mobilized to this area in response to the viral infection (Fig. 7D). Although numerous IgT$^+$ cells could be detected in the midgut/hindgut region (Fig. 7E), the numbers were not affected by the infection with IPNV (Fig. 7F).

3.7. CD3$^+$ cell mobilization in response to IPNV along the digestive tract

The presence of CD3$^+$ cells was also evaluated along the digestive tract, using a specific monoclonal antibody against this T cell marker (Boardman et al., 2012). Only a few scattered CD3$^+$ cells were found in the foregut in either control (Fig. 8A) or infected fish (Fig. 8B). On the other hand, many CD3$^+$ cells were found in the pyloric caeca region, mostly as IELs (Fig. 8C). When fish were infected with IPNV, the number of CD3$^+$ cells detected in this region strongly increased (Fig. 8D). This was also the case for the midgut/hindgut region. The number of CD3$^+$ cells present in control fish (Fig. 8E) increased in response to IPNV (Fig. 8F) in this region.

4. Discussion

Although leukocytes can be found all along the digestive tract in rainbow trout (Ballesteros et al., 2013), most studies concerning its immune regulation have focused on the posterior segments exclusively (Rombout et al., 2011). In the current work, we have compared the effects of viral infection to those elicited by oral DNA vaccination along five segments of the digestive tract, focusing on studying the transcription of several genes important for mucosal immunity. First we studied the transcription levels of CK9,

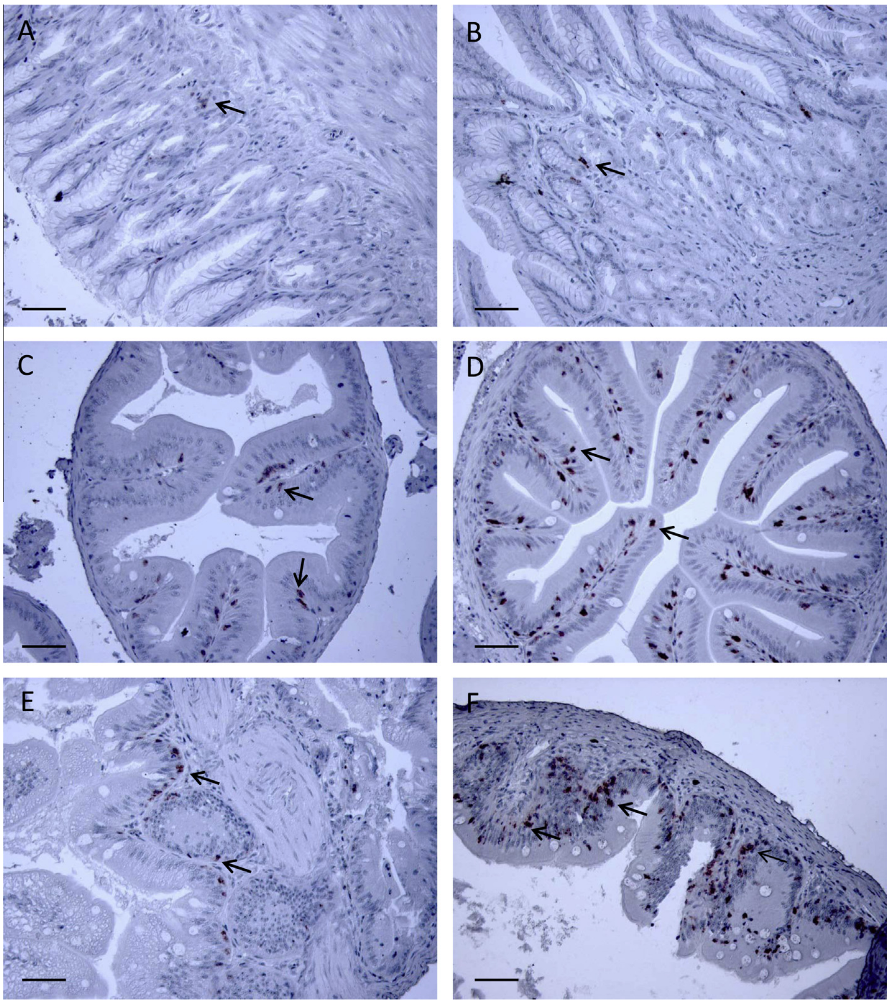

Fig. 8. CD3^{+} cell detection in the different gut segments in IPNV-infected fish and mock-infected fish. Immunohistochemical detection of trout CD3^{+} cells in the foregut (A and B), pyloric caeca (C and D) or midgut/hindgut region (E and F) of mock-infected fish (A, C, E) or IPNV infected fish (B, D, F). Trout were infected with IPNV as described in the legend of Fig. 1 and sampled at day 7 post-infection. Arrows indicate examples of positive cells. Bar: 200 μm.

CK10, CK11 and CK12 chemokines. These chemokines are regulated in response to VHSV infection in mucosal tissues such as gills or skin (Montero et al., 2011). All of them were significantly regulated in response to both the virus and the DNA vaccine, although important differences were observed in these two groups and among the different segments. These results suggest on one hand that the capacity of the different segments to recruit leukocytes is different; and on the other hand, that the virus and the DNA vaccine trigger different immune mechanisms. It might also be possible that the virus and the vaccine do not replicate the same way in the different segments. Upon oral DNA vaccination with the pcDNA-VP2 plasmid, transcription of VP2 can be detected in all segments, although at significantly higher levels in the first three segments (Ballesteros et al., 2013). However, when fish are infected with IPNV, viral transcription levels at day 7 were not significantly different among the different gut segments. Therefore, it seems that the epithelial cells in the different segments, mostly responsible for the production of chemokines, are sensing stimuli in different ways along the different segments. These differences between stimuli and among segments were also visible when the transcription of chemokine receptor genes, MHC-II or TNF-α was analyzed. Indeed IPNV replication is an event much more complex than vaccination, because the virus elicits at the same time a rapid immunological response and damages to the implicated tissues. Furthermore, other structural proteins different that VP2 are known to play an important role in viral pathogenesis (Pedersen et al., 2007). To date, the role of intestine in IPNV infection has been scarcely approached under molecular or physiological point of view. One of the few studies performed in this sense in salmon demonstrated that IPNV can translocate across the intestinal epithelium, eliciting rapid changes in active transport after only 90 min of exposure to IPNV, significantly modulating the barrier function of the intestinal epithelium in the midgut/hindgut region (Sundh et al., 2011). Interestingly, when we evaluated the effects of oral DNA vaccination on the transcription of Ig genes and B cell related transcription factors (Ballesteros et al., 2013), the only segment that significantly responded to the vaccine was the pyloric caeca region. In the current study, however, significant up-regulations of the different genes (chemokines, chemokine receptors, MHC-II or TNF-α) were observed in all the other segments, strongly suggesting that cells different than B cells are playing an important role in mucosal immunity in these other segments. This seems the case even in the stomach, because even though no B cells are visible (Ballesteros et al., 2013), significant CK9, CK10, CK11 and CCR7 up-regulations were observed. In this work, we also report the identification of CD3^{+} T lymphocytes in the foregut, pyloric caeca and midgut/hindgut area. A previous study had already established that T cells were present as IELs in the posterior rainbow trout gut segments (Bernard et al., 2006), however, this is the first report of CD3^{+} T cells in the foregut and pyloric caeca regions. In sea bass, the expression of TCR-β had already been demonstrated by PCR in the anterior segment of the digestive tract (Picchietti et al., 2011). Furthermore, it might be possible that additional cell types such as dendritic cells or NK cells, for which no specific markers are available yet in rainbow trout, are being recruited to the mucosa in response to these chemokines and through the action of these chemokine receptors. CCR7, for example, is strongly expressed in mature dendritic cells in mammals (Sallusto et al., 1998), where it is known to mediate their migration of to the digestive tract (Jang et al., 2006).

Because we had previously established that IgM^+ and IgT^+ cells are recruited exclusively in the pyloric caeca region after oral DNA vaccination (Ballesteros et al., 2013), we now studied the homing of leukocytes to the different gut segments in response to IPNV infection. In this case, although the mobilization of IgM^+ and IgT^+ cells to the pyloric caeca was still massive, an infiltration of both types of B cells was also observed in the foregut. Surprisingly, homing of B cells to the posterior segments was not observed. Previous studies in rainbow trout have demonstrated homing of IgT^+ cells to the posterior gut segments in response to the intestinal parasite *Ceratomyxa shasta* 3 months post-infection (Zhang et al., 2010). It seems probable that important differences in leukocyte homing are induced by different pathogens, whereas it might also be possible that it takes longer for B cells to colonize the posterior segments. Interestingly, the homing of $CD3^+$ cells in response to IPNV infection was also visible in the pyloric caeca and the midgut/foregut regions. Although a homing capacity has been established for mammalian intestinal T IELs (Buzoni-Gatel et al., 1999; Kim et al., 1997), this was not visualized in rainbow trout intramuscularly infected with VHSV (Bernard et al., 2006). Thus, our study constitutes the first report of teleost mucosal T cell homing in response to a viral infection.

In summary, IPNV bath infection in rainbow trout modulated the transcription of CK9, CK10, CK11, CK12, CCR7, CCR9, CCR9B, MHC-II and TNF-α in the digestive tract. Oral DNA vaccination against IPNV also modulated these genes, although important differences were observed concerning the gut segments where these modulations took place and the effects themselves. On the other hand, we have demonstrated that the IPNV infection mobilized IgM^+ and IgT^+ cells to the foregut and pyloric caeca regions, as well as $CD3^+$ T cells to the pyloric caeca and midgut/hindgut regions. These studies contribute to a better understanding of antiviral mucosal immunity in teleost.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.12.009.

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Tanto la vacuna de DNA “pcDNA-VP2” por vía oral como el virus IPNV tienen la capacidad de desencadenar una respuesta inmune completa en los diferentes segmentos del tracto intestinal. En el caso de la estimulación con el virus IPN se observó la secreción de IgM^{+} e IgT^{+} a lo largo del intestino; sin embargo, todavía hay escaso conocimiento acerca de cómo la inmunidad intestinal se encuentra regulada, además de la ubicación de células B, a lo largo del tracto digestivo y su papel ante un estímulo local.

Por esta razón se planteó el siguiente trabajo, centrado en la presencia de linfocitos B en el intestino de trucha arco iris y la activación de células IgM^{+} e IgT^{+} tras la vacunación oral frente a IPNV.

1.4 Ciego pilórico como principal órgano en el reclutamiento de células B IgM⁺ e IgT⁺ en truchas vacunadas oralmente con pcDNA-VP2

A continuación, debido a la respuesta inmunológica observada en los diferentes segmentos del intestino de truchas vacunadas, se profundizó en el estudio de la respuesta inmunológica celular a los 10 días post tratamiento; los ensayos se centraron en la observación de la distribución de posibles células B, mediante técnicas de inmunohistoquímica y en el análisis de la transcripción de genes codificantes de Inmunoglobulinas y factores de transcripción (como Blimp1 y Pax5) implicados en la maduración de las células B, mediante RTqPCR. Los genes correspondientes a Blimp1 y Pax5 son útiles en el estudio de las células B a lo largo del tracto digestivo de la trucha. Pax5 es un factor de transcripción expresado en las células B células y este es sub-expresado cuando se produce la maduración de las células B debido a la inducción del gen Blimp1, que es un represor transcripcional.

Diseño experimental:

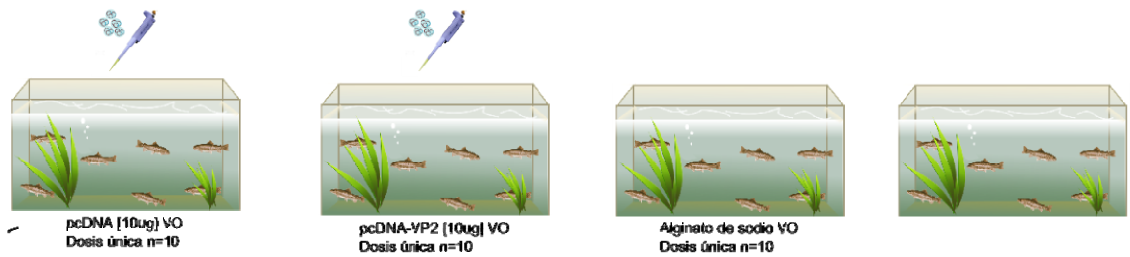

Figura 1: Diseño experimental

Truchas arco iris de aproximadamente 10-20 g, 12 cm de longitud

- Grupo de peces (n=10) vacunados con 10 µg de pcDNA-VP2 por vía oral "vacunados".
- Grupo de peces (n=10) vacunados con 10 µg de pcDNA por vía oral "plásmido vacío".
- Grupo de peces (n=10) Control de alginato de Sodio "Control alginato".
- Grupo de peces (n=10) sin tratamiento. Control

Tiempos de muestreos: 10 días post-tratamiento. 6 individuos muestreados.

Muestras: Intestino dividido en cinco segmentos: (esófago, estómago, intestino ciego pilórico, medio y posterior)

Métodos: Extracción de RNA total utilizando TriZol, síntesis de cDNA, PCR cuantitativa a tiempo real, Técnicas de inmunohistoquímica, Perfusión.

Resumen:

En peces vacunados por vía oral, observamos un aumento en el número de IELs en el ciego pilórico, además de una regulación de genes de IgM, IgT, IgM de membrana y el factor de transcripción Blimp1; los cuales se correlacionan con la transcripción del antígeno VP2 de la vacuna.

La cuantificación de los niveles de expresión de los factores de transcripción Blimp1 y Pax5 en peces perfundidos y sin ningún tipo de tratamiento, se realizó con el fin de conocer los niveles basales propios del tejido intestinal para estos genes, sin interferencia de la expresión producida por los leucocitos de sangre periférica (PBLs).

En resumen, las expresiones de los genes Pax5 y Blimp1 no han sido correlacionadas entre ellos; aunque la sobre-regulación del gen Pax5 en el ciego pilórico en respuesta a la vacunación, es debida a un aumento en las células plasmáticas, y por ende, sus altos niveles de expresión observados son debidos a la expresión de las células plasmáticas fácilmente difundidas por la alta irrigación del tejido, mientras que la sobre regulación de Blimp1 podría indicar la maduración local (en el propio tejido intestinal) de las células plasmáticas como respuesta a la vacunación.

Otro hallazgo importante es la presencia de células B en el tejido adiposo que rodea el ciego pilórico y su capacidad para responder ante una estimulación oral.

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PLOS ONE

The Pyloric Caeca Area Is a Major Site for IgM$^+$ and IgT$^+$ B Cell Recruitment in Response to Oral Vaccination in Rainbow Trout

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Abstract

Although previous studies have characterized some aspects of the immune response of the teleost gut in response to diverse pathogens or stimuli, most studies have focused on the posterior segments exclusively. However, there are still many details of how teleost intestinal immunity is regulated that remain unsolved, including the location of IgM$^+$ and IgT$^+$ B cells along the digestive tract and their role during the course of a local stimulus. Thus, in the current work, we have studied the B cell response in five different segments of the rainbow trout (*Oncorhynchus mykiss)* digestive tract in both naïve fish and fish orally vaccinated with an alginate-encapsulated DNA vaccine against infectious pancreatic necrosis virus (IPNV). IgM$^+$ and IgT$^+$ cells were identified all along the tract with the exception of the stomach in naïve fish. While IgM$^+$ cells were mostly located in the lamina propria (LP), IgT$^+$ cells were primarily localized as intraepithelial lymphocytes (IELs). Scattered IgM$^+$ IELs were only detected in the pyloric caeca. In response to oral vaccination, the pyloric caeca region was the area of the digestive tract in which a major recruitment of B cells was demonstrated through both real time PCR and immunohistochemistry, observing a significant increase in the number of both IgM$^+$ and IgT$^+$ IELs. Our findings demonstrate that both IgM$^+$ and IgT$^+$ respond to oral stimulation and challenge the paradigm that teleost IELs are exclusively T cells. Unexpectedly, we have also detected B cells in the fat tissue associated to the digestive tract that respond to vaccination, suggesting that these cells surrounded by adipocytes also play a role in mucosal defense.

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Introduction

Mucosal immunity in fish has recently become a broadly explored field of research, mainly busted by the need for oral vaccination strategies. Despite this, there are many details of the regulatory and functional aspects of intestinal immunity which are still unknown. Moreover, as many of the features of the mucosal immune system present in mammals such as Peyer's patches or IgA are not found in fish, very few assumptions can be established [1].

Although the structures and segments present in the digestive tract show significant differences among the diverse teleost species, a general division into three main segments has been established and was excellently reviewed by Rombout *et al.* [1]. The first segment or foregut is where the food protein uptake appears to take place, with enterocytes acting as absorptive cells. This segment includes the esophagus and a defined stomach, present in salmonids and not clearly defined in some other fish species such as cyprinids. The second segment is characterized by a strong uptake of macromolecules and enterocytes containing large supranuclear vacuoles, contains the midgut and may include a variable number of pyloric caeca (pyloric appendages) near the pylorus. Fish caeca, present in species such as salmonids, are an adaptation to increase gut surface area, contributing to a higher macromolecule uptake than that of the rest of the digestive tract. Finally, the third segment is the hindgut in which enterocytes are thought to have an osmorregulatory function, and includes an anal region that in certain species can constitute a proper rectum separated by valves.

Some previous studies have investigated diverse properties along the teleost digestive tract such as its absorption capacity, but the importance of each gut segment in terms of immunity has not been properly addressed to date [2–4]. Furthermore, most studies concerning the immune responses of the digestive tract, conducted upon oral or immersion stimulation, have been focused on the second gut segment, even though it has been in many occasions misnamed as hindgut when it was really referring to the second segment [1]. These posterior segments have often been used to define what we currently know concerning the presence of lymphoid populations in the digestive tract of teleost fish. Scattered lymphocytes have been observed both in the lamina propria (LP) or residing between epithelial cells. These last cells, designated as intraepithelial lymphocytes (IELs), have been observed in different species such as rainbow trout [5], carp [6] or sea bass [7]. All these

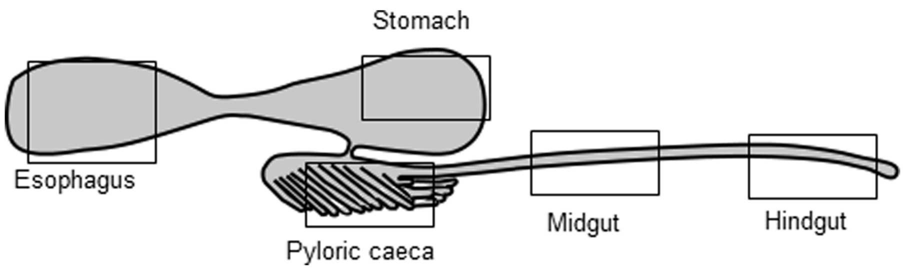

Figure 1. Gut segments used in this study. Schematic model illustrating the different segments in the trout digestive tract used in this study.
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studies suggested that the IELs were Ig-negative T cells. Concerning the presence of B cells in the digestive tract, strong differences are obtained among the different species. Rather than to actual differences, it has been speculated that some of these number variations are due to technical problems, including differences in antibody affinity or reactivity in the case of immunohistochemical studies or problems in the release of B cells from the connective tissue in the case of lymphocyte isolation from the gut segments. For example, abundant numbers of IgM^{+} B cells were found in the LP of carp through immunofluorescence, while isolated leukocytes from carp intestine mainly consisted in IgM^{-} cells [6]. In rainbow trout, the numbers of IgM^{+} cells reported to date in the final gut segments has always been low [8,9]. To the light of recent discoveries, very little attention has been paid in the past years to the role of IgM^{+} cells in the teleost digestive tract. The discovery of IgT, a novel fish-specific Ig subtype [10], also named IgZ in some species [11] and the revelation that IgT^{+} cells constituted an independent B linage specialized in mucosal immunity [8,10], may have led to the incorrect thinking that mucosal IgM specific responses do not significantly contribute to local pathogen defense. However, even if IgT plays an important role in mucosal immunity, some teleost species, such as channel catfish, do not seem to have an IgT or IgZ-like sequence but do present a specific mucosal response [12,13]. Therefore, it seems probable that local IgM mucosal responses play an important role together with IgT responses, as in mammals both IgA and IgM-specific plasma cells have been proved to play a combined role in mucosal immunity [14,15].

In the present study, we have investigated the distribution of B cells through the digestive tract by both real time PCR analysis of Ig mRNA levels and immunohistochemistry in physiological conditions as well as after oral immunization. For the later, we have used a previously described strategy for the oral administration of a DNA vaccine coding for the infectious pancreatic necrosis virus (IPNV) VP2 antigen after alginate encapsulation. This vaccination strategy seemed as a good model as it is capable of inducing a systemic antibody response and protection against viral challenge [16]. Our results reveal important differences in B cell presence among the different segments of the digestive tract, and surprisingly point to the pyloric caeca region as to the segment in which the recruitment of both IgM^{+} and IgT^{+} cells is most significant. Along with this difference, we have also found important variations in the transcription levels of the secreted and membrane forms of IgM, as well as the transcription factors involved in B cell maturation, Blimp1 and Pax5. Finally, we describe the presence of B cells embedded within the fat tissue associated to the digestive tract, detecting a regulation in response to local stimulation that suggests their implication in the local immune response.

Materials and Methods

Ethics Statement

The experiments described comply with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals and were previously approved by the INIA Ethics committee.

Fish

Healthy specimens of rainbow trout (Oncorhynchus mykiss) of approximately 10–20 g were obtained from Centro de Acuicultura El Molino (Madrid, Spain). Fish were maintained at the Centro de Investigaciones en Sanidad Animal (CISA-INIA) laboratory at 14°C with a re-circulating water system, 12:12 hours L:D photoperiod and fed twice a day with a commercial diet (Skretting, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks and during this period no clinical signs were ever observed. In addition, two pools of 5 fish were tested by standard methods to confirm the absence of any salmonid virus by isolation using BF cells [17].

Tissue Collection in Naïve Fish

To characterize the B cell population along the trout digestive tract unhandled naïve trout were used. Five different segments of the digestive tract (esophagus, stomach, pyloric caeca, midgut and hindgut) were directly removed from three individual fish previously sacrificed by MS-222 overdose and were included in Bouin's solution for further inmunohistochemical analysis. A schematic representation of the segments of the digestive tract used in this study is included in Fig. 1. To further investigate Blimp1 and Pax5 levels in the different gut segments, their levels of transcription were also analyzed in blood depleted (buffer perfused) naïve fish as well as in peripheral blood leukocytes (PBLs). For this purpose, blood was extracted from the caudal vein with a heparinized needle. Subsequently, a transcardial perfusion was conducted to remove the circulating blood from the tissues. Heart was cannulated through the ventricle into the bulbus arteriosus for perfusion with 30 ml of teleost Ringer solution pH 7.4 containing 0.1% procaine, using a peristaltic pump at a constant flow rate of ~5 ml per min, whereas the atrium was cut to drain the blood out of the circulatory system. After perfusion, the gut segments were sampled as above (Fig. 1) and included in Trizol (Invitrogen) for further RNA extraction. Blood was diluted 10 times with of Leibovitz medium (L-15, Invitrogen) supplemented with 100 I.U./ml penicillin, 100 μg/ml streptomycin, 10 units/ml heparin and 2% fetal calf serum (FCS, Invitrogen). The resulting cell suspension was placed onto 51% Percoll density cushions which were centrifuged at 500×g for 30 min at 4°C. The interface cells were collected and washed at 500×*g* for 5 min in L-15 containing 0.1% FCS. Cells were then resuspended in Trizol for RNA extraction.

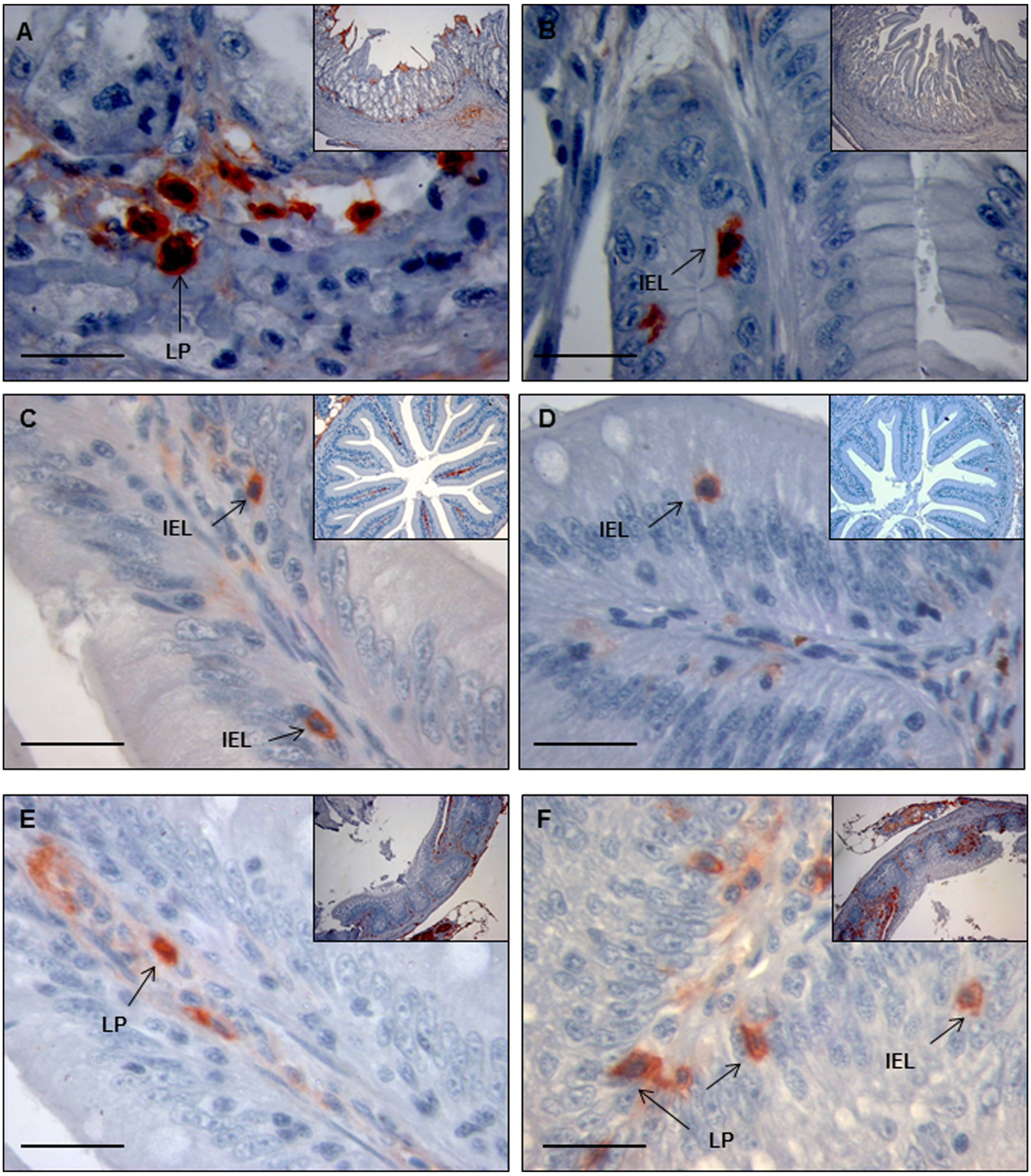

Figure 2. Inmunohistochemical detection of trout IgM$^+$ cells and IgT$^+$ cells in different segments of the digestive tract of naïve fish. Detection of IgM$^+$ cells in the foregut (A), pyloric caeca (C) and midgut (E) segments revealed that IgM$^+$ cells are usually located in the LP, with some IEL IgM$^+$ in the pyloric caeca. Arrows show examples of LP lymphocytes (LPL) and IELs. Detection of IgT$^+$ cells in the foregut (B), pyloric caeca (D) and midgut (F) segments showed that most IgT$^+$ cells where located between enterocytes, as IELs. The figures show a magnification image and an insert figure of the general location in which the detail was observed. Bar: 100 µm.
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Oral Immunization Procedure and Sampling

The pVP2 IPNV vaccine in which the IPNV VP2 gene was cloned into the pcDNA3.1/V5/His-TOPO plasmid (Invitrogen) under the control of the immediate-early CMV promoter was prepared as previously described [16,18,19]. The empty pcDNA3.1/V5/His-TOPO plasmid (pcDNA) was used as a control in the immunization procedures.

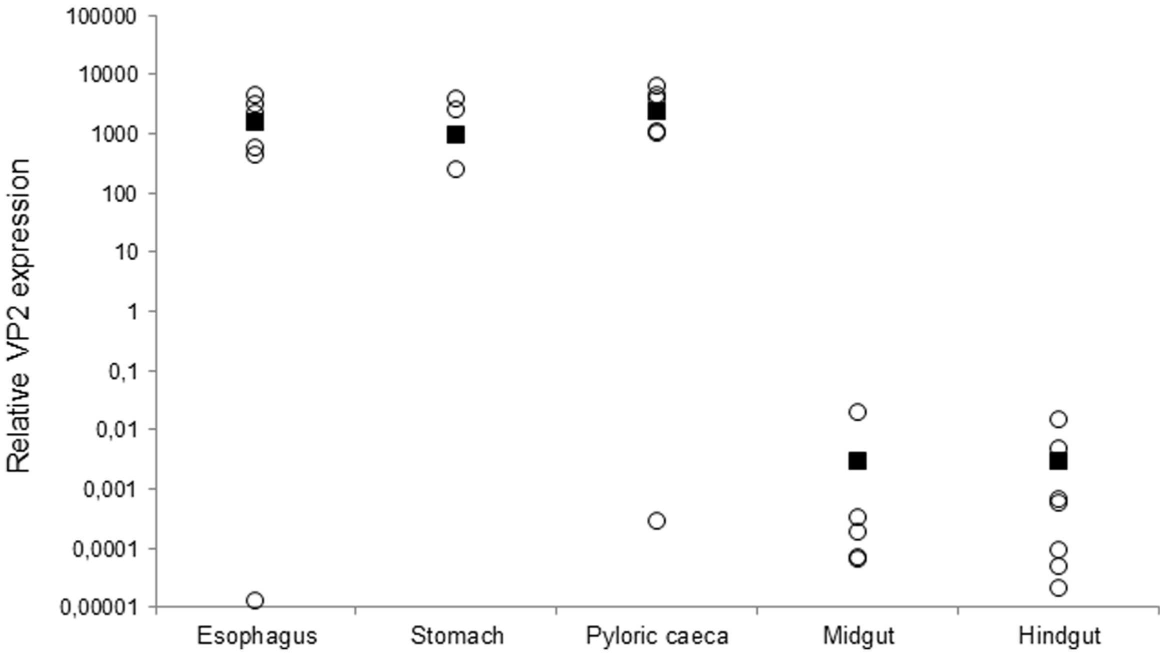

Figure 3. VP2 transcription in the different segments of the digestive tract of vaccinated fish. Data are shown as relative transcription levels of VP2 normalized to the transcription of the house-keeping gene EF-1α in fish orally-vaccinated with a VP2 DNA vaccine at day 10 post-vaccination. Open circles represent relative transcription levels from individual fish, whereas black squares represent mean values in each segment.
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The procedure to encapsulate the DNA in microspheres has also been previously described [16]. Briefly, 2.5 ml of 3% (w/v) sodium alginate were mixed with 1.5 ml of pcDNA-VP2 (1 mg/ml) and the mixture stirred at 500 rpm for 10 min. This solution was then added to an Erlenmeyer flask containing 100 ml of paraffin oil and 0.5 ml Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared adding 2.5 ml of 0.15M $CaCl_2$ drop-by-drop to the emulsion and stirring for 2 h at 900 rpm and were then collected by centrifugation at $1000\times g$ for 10 min. They were washed twice with 70% ethanol, lyophilized and stored at 4°C until used.

For the immunization experiments, trout were divided into three different groups. One group was orally vaccinated with 10 µl of the vaccine microsphere suspension containing 10 µg of pVP2, while a second group received 10 µg of the pDNA empty plasmid diluted in 10 µl of a microsphere suspension. Finally, a third group received the same volume of microsphere suspension with no DNA. Vaccination was performed with an automatic pipette with a 20 µl tip which was introduced into the mouth of each trout, supporting the tip end at the entrance of the digestive tract. The water-quality parameters were maintained at optimal levels and equal in all tanks.

At day 10 post-vaccination, six fish from each group were sacrificed by MS-222 overdose and the esophagus, stomach, pyloric caeca, midgut and hindgut collected and included in Trizol for RNA extraction. This time point was chosen because previous studies had determined the highest transcription levels of the VP2 viral antigen in the midgut segment at this time (data not shown). Four additional fish in each group were sacrificed (control and vaccinated fish) and sampled for immunohistochemistry.

The levels of Ig transcription in the adipose tissue of vaccinated and mock-vaccinated fish were also studied. For this, the large accumulation of white adipose tissue located over the digestive tract was sampled in some individuals and included in Trizol for RNA extraction.

Immunohistochemistry

Segments from the digestive tract obtained from control and orally immunized fish were fixed in Bouin's solution for 24 h, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5 µm. After dewaxing and rehydration, some sections were stained with hematoxylin–eosin in order to determine the levels of infiltration, apparent damages or pathological changes. A second set of sections was subjected to an indirect immunocyto-chemical method for detection of trout IgM and IgT using monoclonal antibodies kindly donated by Dr. Kurt Buchmann from the University of Copenhagen and Dr. Karsten Skjoedt from the University of Southern Denmark (Denmark) [20,21]. These antibodies recognize both the membrane and the secreted forms of these Igs. Endogenous peroxidase was inhibited after rehydration by 10 min incubation in 3% H_2O_2 in PBS. After a heat induced epitope retrieval in Tris-EDTA buffer pH 9.0 (800 w for 5 min and 450 w for 5 min in a microwave oven) [22], the sections were pre-incubated in two different blocking solutions consisting of 2% BSA (bovine serum albumin; Sigma-Aldrich) in TBT (Tris buffer with 0.02% tween 20) at room temperature for 30 min, and 10% normal goat serum in TBT for 30 min. Then, sections were incubated with primary antibody solution overnight at 4°C. Monoclonal mouse anti-trout IgM and monoclonal mouse anti-trout IgT were used in dilutions of 1:150 and 1:300, respectively. Following this incubation, unbound primary antibodies were washed off using TBT. The tissue was covered with anti-mouse EnVision^{TM} System HRP labeled secondary antibody (Dako) and left for a 30 min incubation period at room temperature. Subsequently, the tissue was washed three times with TBT and then incubated in AEC substrate [0.05M acetic acid buffer (pH 5) with 0.015% H_2O_2 and 0.4 g/l 3-Amino-9-ethylcarbazole (Alfa

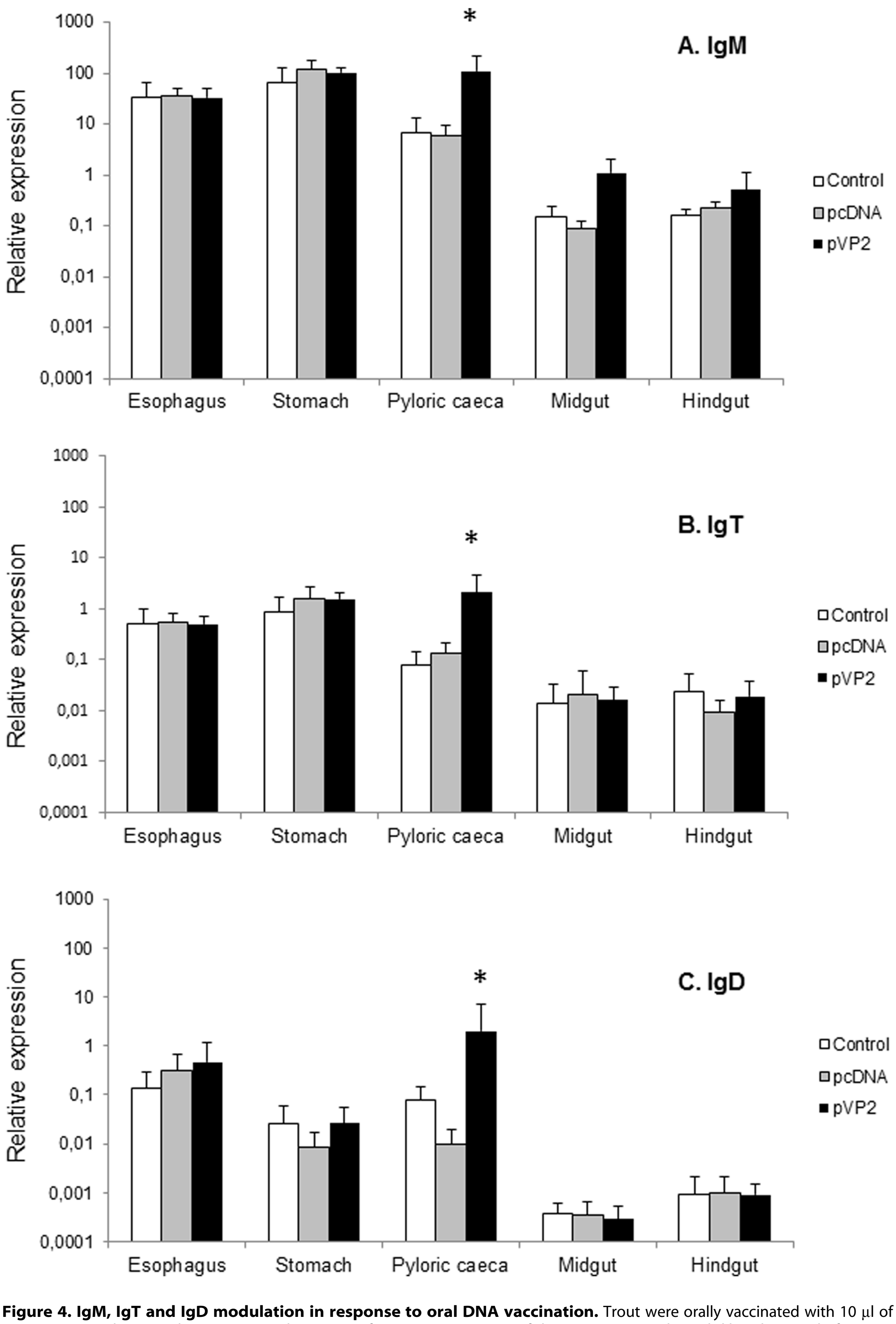

Figure 4. IgM, IgT and IgD modulation in response to oral DNA vaccination. Trout were orally vaccinated with 10 µl of suspension of the vaccine microspheres each containing either 10 µg of pDNA-VP2 or 10 µg of the pDNA empty plasmid diluted in 10 µl of PBS. Finally, a third group received the same volume of an empty microsphere suspension. At day 10 post-vaccination, trout were sacrificed and the different segments of the digestive tract removed for RNA extraction and analysis of immune gene transcription through real time PCR. Levels of IgM (A), IgT (B) and IgD (C)

transcription in the different segments were studied through real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF−1α ± SD (n = 6). The relative significance of differences between fish vaccinated with either the empty plasmid or empty microspheres and vaccinated fish at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as *.
doi:10.1371/journal.pone.0066118.g004

Aesar)] for 15 min and afterwards washed for 4 min in tap water. The specificity of the reactions was determined by omitting the primary antibodies. Mayer's haematoxylin (Dako) was used as nuclear counter stain, and mounting was conducted with Aquamount (Merck). Slides were examined with an Axiolab (Zeiss) light microscope.

cDNA Preparation

Total RNA was extracted from different gut segments or PBLs using a combination of Trizol (Invitrogen) and RNAeasy Mini kit (Quiagen). In summary, tissues were first homogenized in 1 ml of Trizol in an ice bath, two hundred µl of chloroform were then added and the suspension centrifuged at 12000×*g* for 15 min. The clear upper phase was aspirated, mixed with an equal volume of 70% ethanol in diethylpyrocarbonate (DEPC)-treated water and immediately transferred to RNAeasy Mini kit columns. The procedure was then continued following the manufacturer's instructions, performing on-column DNase treatment. Finally, RNAs pellets were eluted from the columns in DEPC- water and stored at −80°C until used.

Four µg of RNA were used to obtain cDNA in each sample using the Bioscript reverse transcriptase (Bioline Reagents Ltd) and

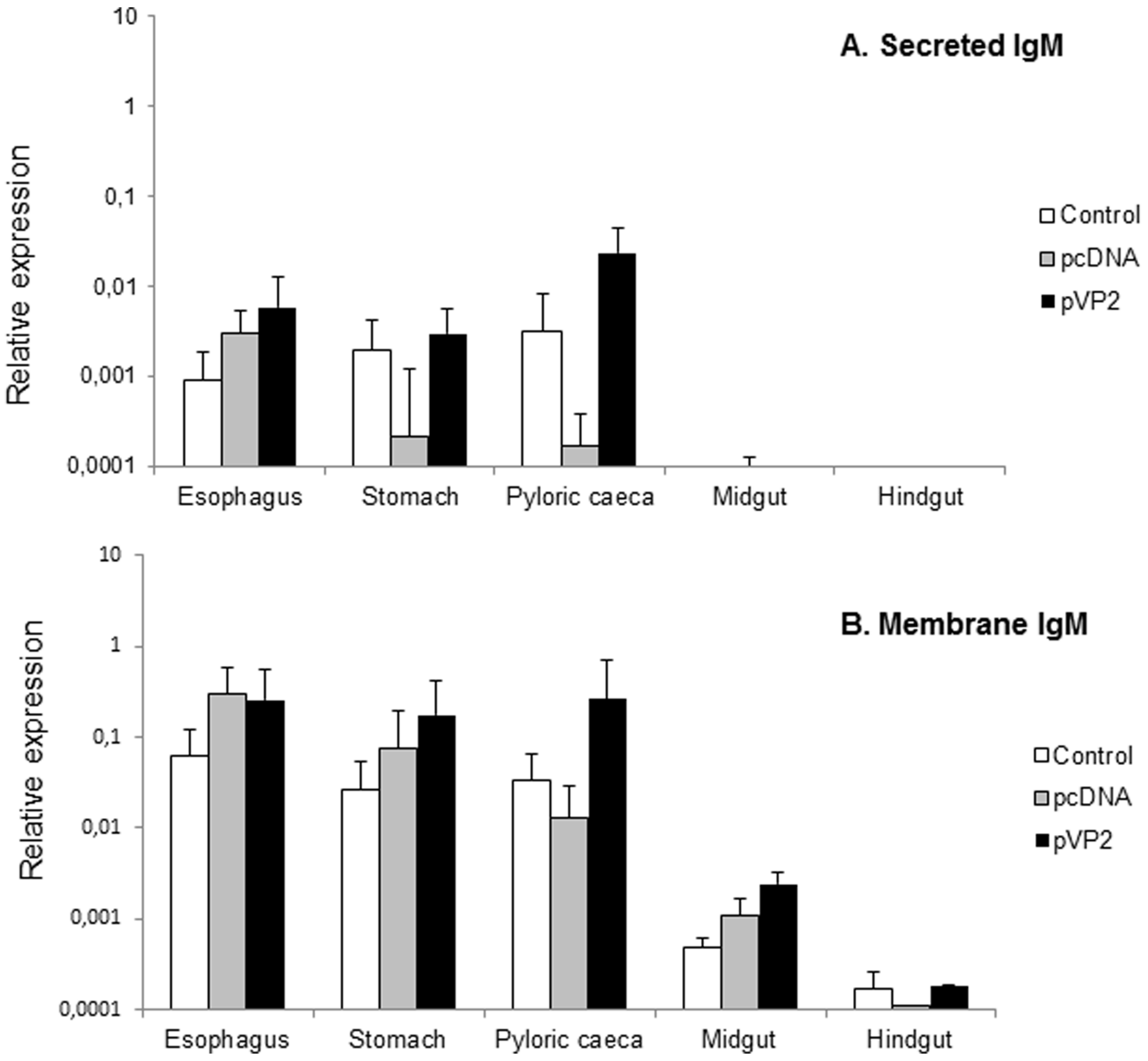

Figure 5. Secreted IgM and membrane IgM modulation in response to oral DNA vaccination. Trout were orally vaccinated and sampled as described in the legend of Figure 4 and levels of secreted IgM (A) and membrane IgM (B) transcription in the different segments were studied through real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α ± SD (n = 6). The relative significance of differences between fish vaccinated with either the empty plasmid or empty microspheres and vaccinated fish at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as *.
doi:10.1371/journal.pone.0066118.g005

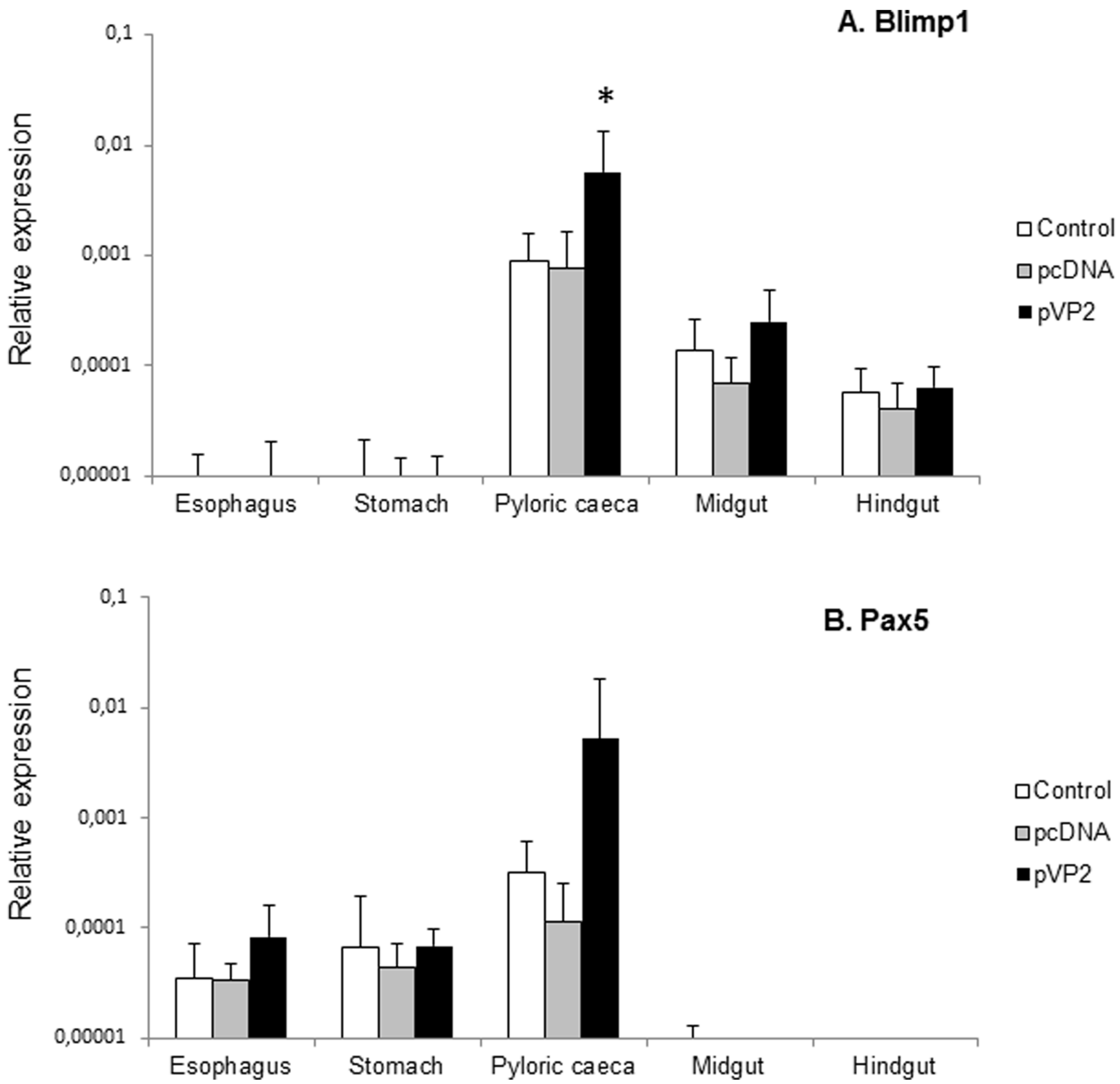

Figure 6. Blimp1 and Pax5 modulation in response to oral DNA vaccination. Trout were orally vaccinated and sampled as described in the legend of Figure 4 and levels of Blimp1 (A) and Pax5 (B) transcription in the different segments were studied through real time PCR. Data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α $\pm$ SD (n = 6). The relative significance of differences between fish vaccinated with either the empty plasmid or empty microspheres and vaccinated fish at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as*.
doi:10.1371/journal.pone.0066118.g006

oligo $(dT)_{12-18}$ (0.5 µg/ml) following the manufacturer's instructions. The resulting cDNA was diluted and stored at $-20°C$.

Evaluation of Immune Gene Expression by Real Time PCR

To evaluate the levels of transcription of different immune genes in the different segments of the digestive tract, real-time PCR was performed with a LightCycler® 480 System (Roche) using FastStart SYBR Green Master mix (Roche). Reaction mixtures containing 5 µl of 2× SYBR Green supermix, 1 µl of each primer (1 mM each) and 2 µl of cDNA template were incubated for 10 min at 95°C, followed by 40 amplification cycles (30 s at 95°C and 1 min at 60°C) and a dissociation cycle (30 s at 95°C, 1 min 60°C and 30 s at 95°C). For each mRNA, gene expression was normalized by the elongation factor 1α (EF-1α) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the EF-1α Ct value from the target Ct as previously described [23]. All the primers used had already been optimized in previous studies [23–25]. Amplifications were performed in duplicate and negative controls with no template were always included in the reactions.

Statistics

Prior to statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro Wilk test. To determine if there was a differential expression in the levels of gene transcription among the different groups, factorial ANOVAs were run followed by Tukey's multiple comparison test for differences between the vaccinated group (pVP2) and the groups vaccinated with the empty plasmid (pcDNA) or mock-vaccinated. For the Pearson correlation, only the data

Table 1. Correlation of VP2 transcription with transcription of immune genes.

	Segments of the digestive tract				
Gene	Esophagus	Stomach	Pyloric caeca	Midgut	Hindgut
IgM	0.484	−0,413	**0.874****	0.084	0.355
IgT	0.411	−0,089	**0.863****	0.198	−0,209
IgD	**0.766***	**0.984****	0.362	−0,335	**0.742***
MembIgM	0.680	−0,383	**0.799***	−0,144	−0,228
secIgM	0.232	0.329	−0,002	−0,215	0.061
Blimp1	0.232	0.132	**0.756***	−0,219	0.447
Pax5	0.060	0.278	0.374	−0,485	0.266

Pearson correlation between the levels of transcription of the VP2 gene and the transcription levels of the different immune genes studied in the different segments of the intestinal tract. N = 6.
*p<0.05;
**p<0.01.
doi:10.1371/journal.pone.0066118.t001

from vaccinated fish in each segment of intestinal tract was used. The correlation was run between the levels of transcription of several immune genes and the levels of transcription of the VP2 gene. In all statistical analysis, *p* values which were less than 0.05 (*) or 0.01 (**) were considered to be significant. All statistics were run in SPSS Version 15.

Results

Identification of IgM$^+$ and IgT$^+$ Cells in the Different Gut Segments Revealed the Presence of IgM$^+$ and IgT$^+$ Trout IELs

Using immunohistochemistry to IgM and IgT to detect B cells in the different gut segments of naïve fish, we were able to identify numerous IgM$^+$ and IgT$^+$ B cells in all the segments except the stomach (data not shown). In the foregut, a strong specific reactivity to IgM was observed in the apical surface of the enterocytes, although most IgM$^+$ cells were present in the loose connective tissue underlying the enterocytes, the LP (Fig. 2A). Although there was little general reactivity against IgT in the foregut, some IgT$^+$ cells were clearly identified (Fig. 2B). These cells were located in the lamina epithelialis interspersed with gut epithelial cells, similarly to mammalian IELs. In the pyloric caeca, numerous IgM$^+$ cells were present in the LP, although in this segment some clear IgM$^+$ IELs were also identified (Fig. 2C). As observed in the foregut, IgT$^+$ cells were not clearly identified in the LP of pyloric caeca, whereas most IgT$^+$ cells were located between enterocytes as IELs (Fig. 2D). In the midgut region, IgM$^+$ IELs were not visualized and IgM$^+$ cells were located in the LP (Fig. 2E). In this segment, however, a high number of IgT$^+$ cells were found both in the LP and as IELs (Fig. 2F). The IgM and IgT reactivity pattern in the hindgut was identical to that observed in the midgut (data not shown). In some cases, these IELs were small round cells similar to other IELs previously described in rainbow trout [5], whereas some other cells contained pseudopodia and a morphology that may suggest an antigen presenting role. These data confirmed that IgM$^+$ and IgT$^+$ cells are constitutively present in the foregut, pyloric caeca, hindgut and midgut of rainbow trout, located mainly in the LP or as IELs depending on the segment.

VP2 is Transcribed along the Digestive Tract Upon Oral Vaccination

Having established the presence of tissue B cells all along the digestive tract, we proceeded to study their response to an oral vaccination protocol that had previously proved effective in controlling viral replication [16]. As a first step, we studied the levels of transcription of the IPNV VP2 gene along the different segments of the digestive tract at day 10 post-vaccination, when the VP2 levels were maximal in the pyloric caeca segment (data not shown). The highest levels of VP2 transcription were observed in the first segments, the esophagus, stomach and pyloric caeca (Fig. 3). Although still detected, the transcription of VP2 was much lower in the midgut and hindgut segments. These results reveal that the DNA vaccine is effectively taken up and transcribed by enterocytes all along the digestive tract.

The Pyloric Caeca is the Most Responsive Segment to Oral IPNV DNA Vaccination

To analyze the differences in B cell response to oral vaccination among the gut segments, we first studied the transcription levels of different Ig genes in vaccinated and control animals mock-vaccinated either with the empty plasmid or with the alginate microparticles alone. IgM, IgT and IgD transcription levels were observed all along the digestive tract in all experimental groups. However, only in the pyloric caeca region, IgM transcription was significantly higher in vaccinated fish than in control groups (either injected with the empty plasmid or with the alginate alone) (Fig. 4A). In the case of IgT and IgD transcription, again, only in the pyloric caeca region the values obtained in the vaccinated group were significantly higher than those obtained in the control groups (Fig. 4B and 4C).

For IgM, specific primers have been previously designed to distinguish between the membrane form and the secreted form of IgM [25]. Using these primers, we observed that, for both the secreted and the membrane form of IgM, transcription levels were always lower in the two more distal segments, the midgut and the hindgut (Fig. 5A and 5B). In the pyloric caeca region, although we observed a higher transcription of the secreted form of IgM in vaccinated animals, the differences were not significantly different than those observed in the group mock-vaccinated with the empty alginate microspheres (Fig. 5A). Surprisingly, these values were significant in comparison to the levels detected in the group vaccinated with the empty plasmid, as there was a marked down-regulation of the transcription levels in this group. The reason for this down-regulation in the levels of transcription of secreted IgM observed in response to the empty plasmid is unknown and should be further investigated. Concerning the levels of transcription of the membrane form of IgM, we found levels of transcription significantly higher in the pyloric region of the vaccinated fish in comparison to those obtained in the control fish (Fig. 5B).

To further understand a possible role of IgM$^+$ B cells along the digestive tract, we also analyzed the levels of transcription of Blimp1 and Pax5. Pax5 is a B cell-specific transcription factor down-regulated through the maturation of B cells due to the induction of the transcriptional repressor Blimp1 [26]. Blimp1 transcription remained almost undetected in the esophagus and the stomach (Fig. 6A). Then the levels increased in the pyloric caeca region, to decrease again in the last two segments. In response to vaccination, it was only in the pyloric region, where differences between the vaccinated group and the control groups were significant. This same response in the pyloric caeca region was observed for Pax5 transcription (Fig. 6B); however, in this case the Pax5 transcription levels in the last two segments remain almost undetected.

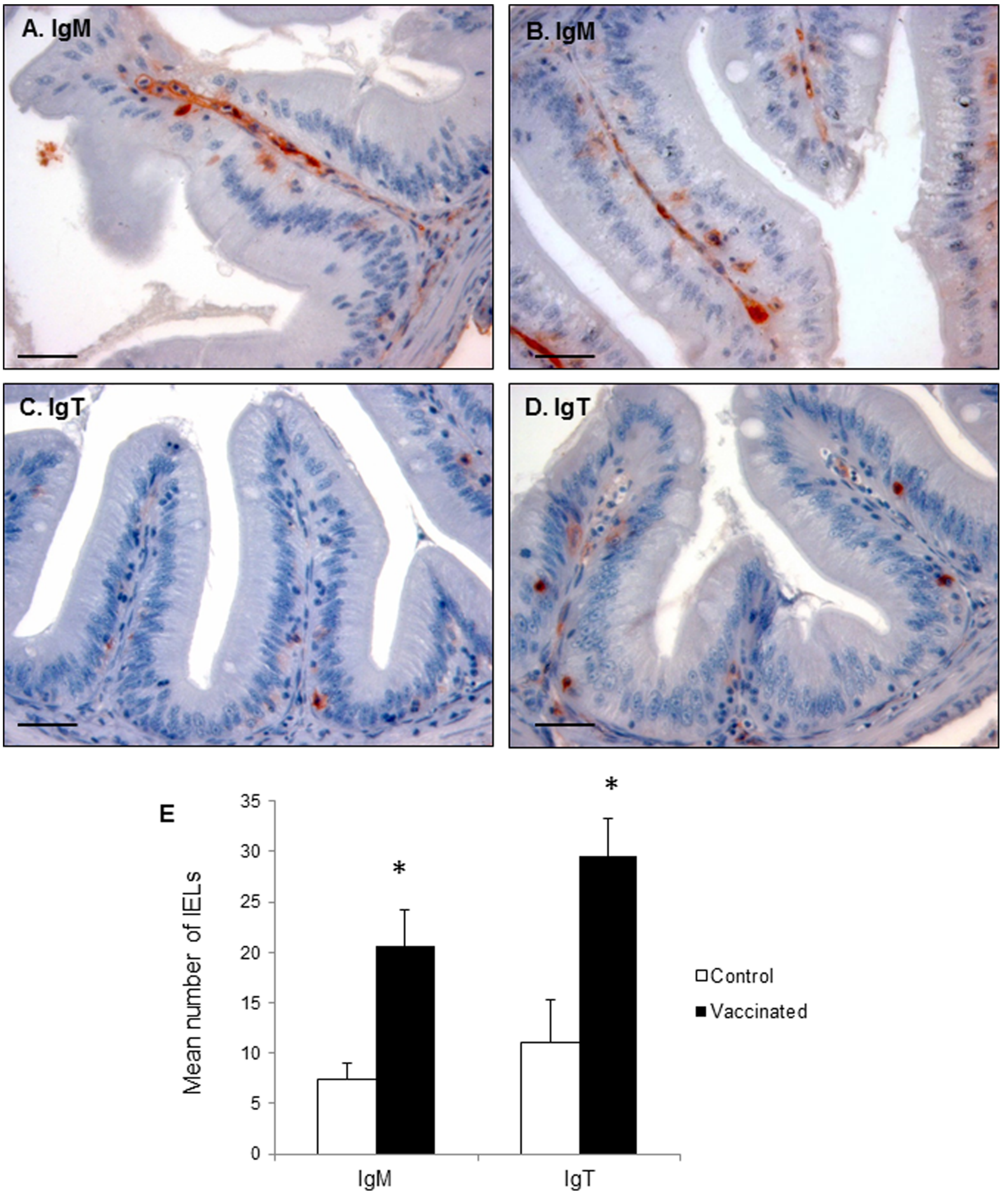

Figure 7. B cell detection in the pyloric caeca after oral DNA vaccination. Immunohistochemical detection of trout IgM^{+} and IgT^{+} cells in pyloric caeca of mock-vaccinated (A, C) and vaccinated (B, D) fish. Trout were orally vaccinated as described in the legend of Figure 4 and sampled at day 10 post-vaccination. Bar: 200 µm. E. Mean number of IgM^{+} and IgT^{+} IELs counted in 35 fields of control and vaccinated fish (400× magnification). The quantification was performed in triplicate in 3 individual fish per group. *Number of cells in vaccinated fish significantly higher than the number of cells in the corresponding controls.
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The Transcription of Genes Related to B Cell Function Correlate with Antigen Transcription

To understand whether the variations in the levels of transcription of the different immune genes analyzed were directly produced in response to vaccine transcription, we conducted a Pearson correlation analysis using the data from vaccinated animals. We found that in the pyloric caeca region there was a significant correlation between VP2 transcription and the transcription of IgM, IgT, membrane IgM and Blimp1 (Table 1). Unexpectedly, there was no correlation of VP2 transcription and

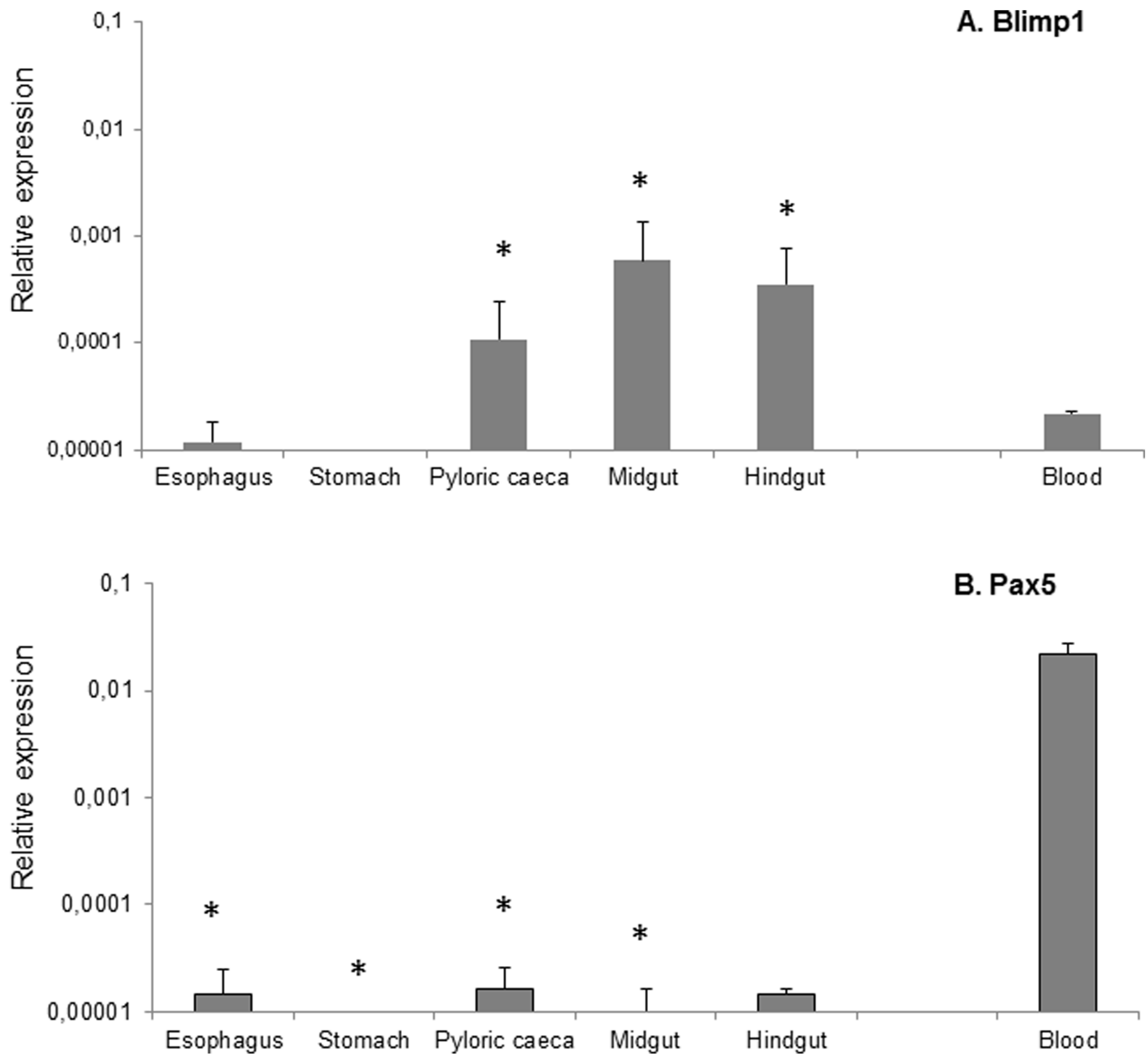

Figure 8. Blimp1 and Pax5 transcription in the gut of perfused fish. Constitutive levels of Blimp1 (A) and Pax5 (B) transcription in different segments of the trout digestive tract after perfusion were compared to transcription levels observed in PBLs. Data are shown as the mean gene expression relative ± SD calculated by the $2^{-\Delta Ct}$, according the formula $\Delta Ct =$ Ct gene -Ct EF-1α. * Levels of transcription in the different gut segments significantly different than transcription levels in the blood.
doi:10.1371/journal.pone.0066118.g008

IgD transcription in this segment; whereas there was a correlation between this two sets of data in the esophagus, the stomach and the hindgut segments. These data further confirm that the up-regulation in the levels of transcription of Ig variants and transcription factors observed in the pyloric caeca are directly produced by the oral vaccination procedure.

B Cells are Mobilized to the Pyloric Caeca Region in Response to Oral Vaccination

To confirm the results obtained through real time PCR that showed that IgM and IgT expression was up-regulated in the pyloric region in response to oral vaccination, and to establish whether this up-regulation was due to a mobilization of cells into this segment or on the other hand to an increased transcription of Ig in individual cells, we analyzed the presence of IgM^+ and IgT^+ cells in both vaccinated and mock-vaccinated animals by immunohistochemistry.

As was described above, the pyloric caeca of naive animals contains IgM^+ cells mainly in the LP, with some cells located between enterocytes as IELs. After vaccination, we observed that the number of IgM^+ IELs significantly increased in this region (Fig. 7A, 7B and 7E). Although even in basal conditions most IgT^+ cells in the pyloric caeca were found as IELs, upon vaccination, we also observed a significant increase in the number of IgT^+ IELs, while still only some IgT^+ cells were present in the LP (Fig. 7C, 7D and 7E).

Blimp1 Levels and Pax5 Levels in Perfused Naïve Fish

Given that both Blimp1 and Pax5 transcription levels were up-regulated in response to oral vaccination in the pyloric caeca, despite being *a priori* negatively correlated, we decided to further investigate the transcription of these factors. For this, the levels of transcription of Blimp1 and Pax5 in the different gut segments were studied in perfused blood-depleted naïve fish, comparing them to the levels observed in PBLs. As already observed in the vaccination trial, Blimp1 was not significantly transcribed in the foregut and stomach; however, mRNA levels were detected in the pyloric caeca, midgut and hindgut segments, at levels significantly higher than those

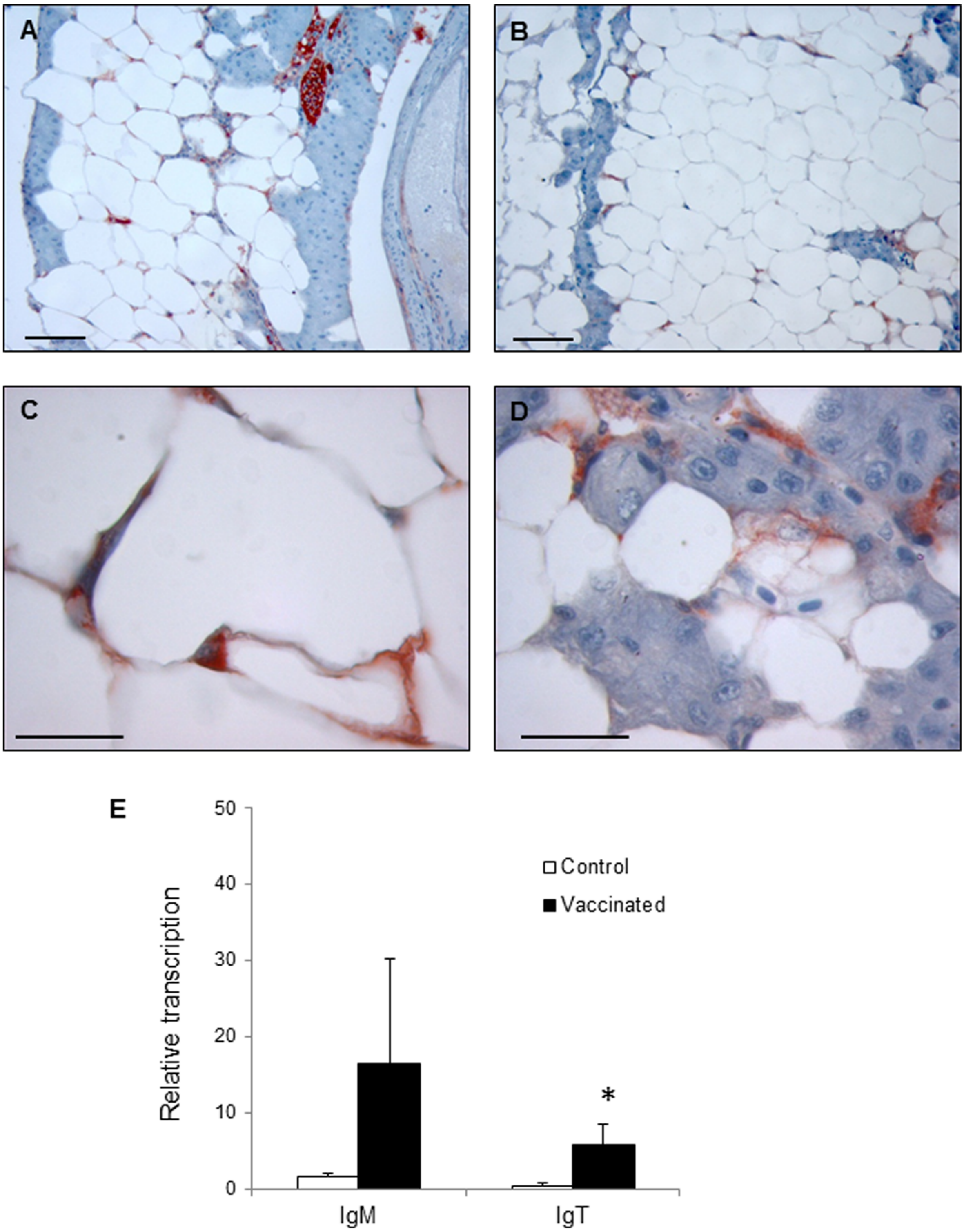

Figure 9. B cells in the adipose tissue surrounding the pyloric caeca. Inmunohistochemical detection of trout IgM+ (A, C) and IgT+ (B, D) cells in the adipose tissue of naïve fish. Bar: 100 µm. E. Levels of transcription of IgM and IgT estimated through real time PCR in control (mock-vaccinated with empty alginate microspheres) and vaccinated fish. Trout were orally vaccinated and sampled as described in the legend of Figure 4 and data are shown as the mean relative gene expression normalized to the transcription of the house-keeping gene EF-1α ± SD. The relative significance of differences between fish vaccinated with the empty plasmid and vaccinated fish at each segment of the digestive tract was determined through a one-way ANOVA and is shown above the bars as*.
doi:10.1371/journal.pone.0066118.g009

observed in PBLs (Fig. 8A). On the other hand, Pax5 was only faintly detected in the esophagus, pyloric caeca and hindgut, whereas the levels of Pax5 transcription in PBLs were 1000 times higher (Fig. 8B). These results, together with the fact that only Blimp1 and not Pax5 levels were correlated to VP2 transcription in the pyloric caeca, suggest that B cells that respond to vaccination in the pyloric caeca have a Blimp1^{high} Pax5^{low} phenotype.

B Cells Located in the Adipose Tissue Associated to the Digestive Tract Respond to Local Stimulation

When examining immunohistological sections of the pyloric caeca region, we observed that the fat tissue surrounding the digestive tract also contained some IgM$^+$ and IgT$^+$ cells (Fig. 9A–D). These IgM$^+$ and IgT$^+$ cells were found in the interstitial space between adipocytes. An important vascularization and endothelial cells were also observed in these adipose structures. To verify whether these immune cells embedded in the adipose tissue also responded to stimulation, we analyzed the levels of transcription of IgM and IgT in this accumulation of adipose tissue associated to the digestive tract obtained from either vaccinated or control fish. Surprisingly, the levels of transcription of both IgM and IgT increased in response to vaccination (Fig. 9E), although in the case of IgM a high variability was observed among the different fish sampled. Therefore, B cells present in the fat tissue surrounding the digestive tract were also responding to oral vaccination.

Discussion

Although different absorption capacities had been reported along different segments of the digestive tract in different teleost species [2–4], their immunological properties had not been properly addressed [1]. In the current work, we have undertaken this task in relation to B cell responses, focusing on B cell mobilization and Ig transcription, and we have demonstrated that concerning B cell responses, the pyloric caeca is the most immunologically active segment of the rainbow trout digestive tract. Its high absorption capacity [1], together with the presence of IgM$^+$ and IgT$^+$ IELs seem as factors that could account for this higher capacity to respond to immune stimuli. Therefore, future studies dealing with immunization in salmonids should take into consideration the immune response elicited in this gut segment.

In naïve fish, both IgM$^+$ and IgT$^+$ B cells were detected in all the segments of the digestive tract, with the exception of the stomach. IgM$^+$ cells were always found in the LP, however, in the case of the pyloric caeca, IgM$^+$ IELs were also identified. On the other hand, IgT$^+$ cells were mostly IELs in all segments and only sometimes they were localized in the LP in the midgut and hindgut regions. In mammals, IELs are primarily T cells with potent cytolytic and immunoregulatory capacities that because of their properties have been cataloged as a mid-way between adaptive and innate immune responses [27]. Accordingly, previous studies in rainbow trout in which IELs had been isolated from the gut, pointed out that those IELs were T cells [5], however, in that study, the pyloric caeca segment was not included nor was the search for IgT transcripts. In the current work, we demonstrate for the first time in fish that IELs include IgM$^+$ and specially IgT$^+$ cells. Similarly, B cells can be found as IELs in other mucosal tissues in mammals and for example IELs of human adenoids and tonsils are enriched in B cells [28]. Taking into account the levels of transcription of the transcription factors Blimp1 and Pax5 in the naïve perfused fish, we can also conclude that the B cells in the pyloric caeca, the midgut and the hindgut have a Blimp1^{high} Pax5^{low} phenotype, in comparison to what we observe in PBLs; therefore, even in basal conditions, B cells in these three segments seem to be more activated than B cells found in the blood. During this activation, Pax5 levels are reduced in part due to the induction of Blimp1 that shifts the Ig expression from membrane to the secreted form [26]. Despite this, in these posterior segments, the transcription of secreted IgM remained almost undetectable. On the other hand, an important level of transcription of secreted IgM was detected in parallel to a Blimp1$^-$ phenotype in the foregut and stomach. The later could suggest the presence of B1 cells in these segments which are known to produce high levels of secreted Ig with low levels of both Blimp1 and Pax5 [29]. Furthermore, the role of Blimp1 and Pax5 in IgT$^+$ B cells is also an unknown issue that should be addressed in the future, giving light to some of our present results. Future work in our group will include a further characterization of the phenotypes of these distinct B cell populations isolated from the different segments.

When fish were orally vaccinated, out of the different segments, the pyloric caeca was the only one which significantly responded to the stimulation through an increased IgM, IgT, IgD, membrane IgM, Pax5 and Blimp1 transcription. Moreover, the transcription levels of IgM, IgT, membrane IgM and Blimp1 in the vaccinated individuals strongly correlated with the transcription of the VP2 viral gene indicating that the up-regulations observed for these genes were a direct consequence of vaccine transcription. Given the fact that Pax5 and Blimp1 have been shown to be negatively correlated and that Pax5 was not correlated to VP2 transcription, we investigated Blimp1 and Pax5 transcription in perfused fish to analyze the response of tissue B cells exclusively, without any input from circulating blood. While Blimp1 levels in the pyloric caeca, midgut and hindgut segments were higher than levels detected in PBLs, the levels of expression of Pax5 in PBLs were 1000 times higher than those obtained in any gut segment. Thus, it seems probable that the up-regulation of Pax5 observed in the pyloric caeca in response to vaccination is a consequence of a higher income of blood into the tissue as a response to vaccination, whereas the up-regulation of Blimp-1 could indicate the maturation of plasma cells locally in response to vaccination.

Our results also reveal the importance of IgM in mucosal responses, challenging the actual line of thought in which IgT seemed to be the Ig subtype mainly responsible for mucosal responses [8]. As more data become available, it seems probable that IgT also plays a role in non-mucosal defense as demonstrated by the fact that IgT expansion in the spleen of virus-infected animals has been observed, revealing that these spleen IgT$^+$ cells contribute to increase IgT plasma levels [30]. Our results suggest that both IgM and IgT together respond at an early stage upon mucosal immunization. It may be possible that different responses are observed as a consequence of different types of immunization, while on the other hand, it may be possible that IgT specific plasma cells take over IgM$^+$ cells only at late times post-immunization, since the previous studies in which IgT$^+$ cells accounted for almost all B cells in the midgut segment were performed in response to a parasite three months after the infection [8].

Finally, our studies have led us to the identification of lymphocyte cells in association with the adipose tissue that surrounds the pyloric caeca. Although the presence of immune cells in diverse adipose structures has been reported in humans and other mammalian models [31], the immune reactions and how their regulation occur in the various environments within the body have been only marginally appreciated in the past, despite the fact that there is a correlation between the presence of fat-associated lymphoid cells and inflammation in obesity [31]. Adipose tissue in mammals is generally separated into visceral and subcutaneous adipose tissue, being the visceral adipose tissue the one which is metabolically and immunologically more active [32]. In mammals, this visceral fat tissue refers to adipose within the peritoneal cavity, including depots such as the gonadal fat pad, the omentum, and the intestinal mesentery [33]. Fatty structures such as the omentum are enriched in macrophages and B cells [34], but also possess dendritic cells [35] and NKT cells [36]. In our studies, we have identified both IgM^{+} and IgT^{+} B cells in the interstitial space between adipocytes and we have demonstrated that the levels of transcription of both Ig can be regulated in response to vaccination in this area. In mammals, although B1 B cells predominate in the leukocyte clusters designated as milky spots [37], B2 conventional responses can be found in the omentum as well. For example, peritoneal immunization with bacteria provoked a strong increase in the number and size of the milky spots [34]. These milky spots and a correlated IgG production are also produced in mice lacking spleen, lymph nodes, and Peyer's patches [38]. A more recent paper revealed that, after a gammaherpesvirus infection, immune aggregates within the omentum not only expand in size and number but also contain virus-infected cells [39]. Furthermore, in this same study, a germinal-center B cell population appeared in the omentum of infected animals with earlier kinetics and greater magnitude than that observed in the spleen. In all these experiments, the immunization was performed through intraperitoneal injection; however, not many studies have demonstrated a response of fat-associated immune cells upon oral immunization [38]. All these results point to an important role of fat-associated lymphoid structures in both innate and adaptive immune responses. Having identified these fat-associated immune cells in teleost fish, future studies should be done to determine their precise role in immunity.

In summary, in this study we describe the presence of IgM^{+} and IgT^{+}B cells in the foregut, pyloric caeca, midgut and hindgut of trout, identifying IgM^{+} and IgT^{+} IELs for the first time in fish. Upon oral immunization, we have demonstrated that the number of IELs increases in the pyloric caeca region along with an up-regulation of IgM, IgT, membrane IgM and Blimp1 transcription that strongly correlates with the transcription of the vaccine antigen. Furthermore, we describe the presence of B cells in the adipose tissue associated to the pyloric caeca and their capacity to respond to an oral stimulation. All together, these results provide further insight in the characterization of mucosal B responses in teleost fish.

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Author Contributions

Conceived and designed the experiments: CT SSRS SIP. Performed the experiments: NAB RC BA. Analyzed the data: CT. Contributed reagents/materials/analysis tools: CT SSRS SIP. Wrote the paper: CT SSRS SIP.

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1.5. Herramientas desarrolladas para la ejecución de esta tesis doctoral (Gene2Path)

Durante el desarrollo del trabajo de Tesis y con objeto de facilitar el análisis de datos generados por microarrays fue necesario diseñar un programa informático que denominamos "Gene2Path" y que tiene la capacidad de buscar de forma automática varios genes a la vez en las bases de datos del NCBI para obtener genes ortólogos correspondiente a especies disponibles en las bases de datos de KEGG.

Para trucha arco iris existen pocos datos disponibles en las bases de datos, por lo que se hizo necesario obtener genes ortólogos en especies con mayor información libre, como zebrafish y así poder obtener datos extrapolables para realizar vías metabólicas o "pathways". Estos pueden proveer bastante información relacionada con las interacciones funcionales o relaciones entre los componentes físicos y genéticos de las células. Actualmente existen muchos programas informáticos muy robustos disponibles a través de la web, que buscan pathways en varias bases de datos, no solamente utilizando KEGG; éstos programas están muy bien completados para datos de ratón, rata, humano, zebrafish, entre otros (STARNET 2, Reactome, Cytoscape, Babelomics (http://babelomics.bioinfo.cipf.es), GEPAS (http://www.gepas.org)); pero desafortunadamente no es así para datos de otras especies, como es el caso de la trucha arco iris. Por esta razón, son necesarios programas informáticos más flexibles, eficientes y que provean información.

Este programa Gen2Path nos permitió analizar los genes del experimento del microarray (sección 1.1) para buscar la función biológica descrita de estos genes en rutas. P ara ello, se requirió buscar los genes ortólogos en zebrafish u otras especies. Estos pasos fueron necesarios debido a que muchos de los genes descritos en el microarray, no cuentan con una descripción dentro de alguna ruta o pathway, y además es difícil esta búsqueda debido a que muchas bases de datos con información acerca del genoma y transcriptoma son de carácter privado, por lo cual no puede ser fácilmente consultada.

En resumen, el programa Gene2Path, realiza varias búsquedas automáticas en diferentes bases de datos bajo los siguientes 5 pasos:

1. Busca los IDs sinónimos de genes utilizando las bases de datos del NCBI tales como EST y NUCCORE.

2. Analiza los resultados del microarray utilizando el valor de la intensidad de la señal "gProccesedSignal" en microarrays de un solo canal; para esto se requieren los archivos tanto de los datos del experimento como del grupo control en formato txt para su correcta normalización.

3. Busca automáticamente los genes ortólogos en otras especies a través de las bases de datos del NCBI como Gene, Unigene y Homologene donde se pueden comparar las secuencias de nucleótidos con proteínas. Por otro lado, para los genes sin información u ortología en otras especies, se pueden, identificar las

secuencias de los genes a través de BLASTx utilizando el programa informático BLAST2GO, el programa Gene2Path permite filtrar según la especie de interés por el usuario, organizar la información automáticamente de los archivos con formato xml que arroja el programa BLAST2GO y permite la visualización rápida y fácil de estos archivos.

4. Búsqueda en los pathways reportados en la base de datos de KEGG de los genes involucrados en ellos, los datos de los genes deben corresponder a especies soportadas en KEGG, si esto no es así, se requiere utilizar los genes ortólogos en una especie soportada por KEGG hallados a través del paso anterior. La búsqueda en la base de datos de KEGG se realiza a través del Gene IDs de cada uno de los genes.

5. Se pueden obtener de forma automática todos los genes involucrados en pathways reportados en KEGG al incluir sus secuencias en formato FASTA Para todos los pasos anteriores, no se requieren conocimientos en programación, ya que se ofrece una aplicación web donde todas las consultas pueden ser llevadas a cabo a través de un entorno gráfico. También se proporciona el código fuente para los usuarios que deseen utilizar este programa desde consola. Por otra parte, el código es libre “open-access” por lo que cualquier persona puede descargarlo, instalarlo y si desea modificarlo o mejorarlo.

Se ha confeccionado una página web cuyo tutorial es muy sencillo y didáctico, que permite realizar el proceso con rapidez. El trabajo se ha publicado y el acceso a la página es libre, a través del enlace http://gene2path.cib.csic.es.

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Research Article   Open Access

Gene2Path: A Data Analysis Tool to Study Fish Gene Pathways by Automatic Search of Orthologous Genes

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Abstract

Most of the gene regulation pathways data from biochemical and molecular experiments are drawn from humans or from species commonly used as experimental animal models. Accordingly, the software packages to analyse these data on the basis of specific gene identification codes (IDs) or accession numbers (AN) are not easy to apply to other organisms that are less characterized at the genomic level. Here, we have developed the Gene2Path programme which automatically searches pathway databases to analyse microarray data in an independent, species-specific way. We have illustrated the method with data obtained from an immune targeted rainbow trout microarray to search for orthologous pathways defined for other well known biological species, such as zebrafish, although the software can be applied to any other case or species of interest. The scripts and programme are available and free at the “GENE2PATH” web site http://gene2path.no-ip.org/cgi-bin/gene2path/index.cgi. A user guide and examples are provided with the package. The Gene2Path software allows the automated searching of NCBI databases and the straightforward visualization of the data retrieved based on a graphic network environment.

Keywords: Pathway analysis tool; Microarrays; Species-independent pathway analysis; Orthologous genes

Introduction

The use of high-density microarrays had a significant impact on studies of gene expression, attracting much interest among biologists. Microarray technology have been used to test the expression of thousands of genes in a single experiment, exploiting the ability of messenger RNA (mRNA) to bind specifically to the DNA template from which it was derived. Microarray gene expression screening can identify the genes involved in a given process, as well as predict interactions among thousands of genes by studying genome transcription. Many fields have benefited considerably from DNA microarray technology, such as drug discovery and toxicological research [1,2], as well as human disease diagnosis. However, studies in the field of veterinary sciences have been more restricted due to the lack of their genome sequences. In fish, for instance, most microarray and/or RNAseq studies have been carried out on zebrafish (*Danio rerio)*, which is a well characterized species with large amount of sequenced genome for which commercial arrays are available. However, other economically relevant cultured fish are still far from having their complete genome sequenced, such as turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*). Although some commercial microarrays have recently been made available for some of these species such Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorynchus mykiss* Walbaum); their genome coverage is still far from that of the zebrafish [3].

Several studies have highlighted the importance of presenting microarray data in the framework of documented biological pathways [4,5]. Typically, microarray gene expression experiments produce long lists of genes that are differentially expressed in two different circumstances. The information regarding these pathways is difficult to apply to species that are not well characterized, mainly because most of the actual software packages use species-specific gene identification data (IDs) from a few biological species that cannot handle genomic data for other less well known species.

To circumvent this problem it has been proposed that “....most of the genetics and physiology of the less well-represented species will be similar or comparable with the data of human and laboratory animal species stored in the database” [6]. Thus, ranking pathways in terms of their relevance to a particular phenotype or metabolic route can help researchers focus on a few sets of genes and such an approach may be very useful to answer some biological hypotheses.

However most biologists and veterinarians who are not familiar with simultaneous upload of thousands of data may have some difficulty because both, the need to configure on a local computer and the excessively long computing times required for analyzing several genes at once, are prohibitive.

In this study, we present an open access web server called Gene2Path for analyzing microarrays results and automatic searches of orthologous genes to be associated in pathways.

Therefore, the main objective of the present study was to develop and validate a tool that extrapolates information associated with different pathways across different species. The programme provides a software tool that uses species-independent gene IDs and streamlines that process searching for information regarding pathways in online public databases. The software uses lists of genes in microarrays (IDs), combining pathway information with this microarray data. Other researchers working with less well studied species, such as the chicken, have also faced the same problem when analyzing the pathways associated with the data derived from microarrays. A tool to study a

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chicken-specific reaction to bacterial infection was developed by Pas et al. [6] based on a set of PERL scripts to extract data from databases via internet and using the names of the genes in the microarray. The gene short names and synonyms were extracted from the GO and KEGG databases, and the results were visualized using colour codes when they combined the pathway data with the microarray data.

In our case example rainbow trout-specific pathways are not available. Therefore, we used pathway schemes from other species (such as zebrafish), to obtain orthologous pathways. There are some free programs for the analysis of data from microarrays in an specific pathway mammal, but they mostly work for human, mouse or other models species not for commercial and marine fish. Our Gene2Path program has the so called "Orthology step", which offers the possibility to detect homology between the non annotated genes or sequences of the uncommon specie with other eukaryote genomes and annotated gene IDs.

We are interested in teleost fish virology and immunology, and accordingly we validated this tool by identifying immune and infection-realted signalling pathways for fish species and chose for that the KEGG database (http://www.genome.ad.jp/kegg/).

Infectious pathogens are a serious problem in aquaculture, and salmonid fish viruses are responsible for important losses in rainbow trout and salmon farming, reflected in the intense research into these viruses within the field of aquaculture [7-9]. Infectious pancreatic necrosis virus (IPNV) for example is the aetiological agent of a well characterized acute disease that produces systemic infection and relevant mortality in farmed rainbow trout (*Oncorhynchus mykiss*) and other salmonid species. The mortality of this virus may be as high as 70% in young fish. The virus establishes an asymptomatic carrier state in survivors [10], both in different species of salmonids and in other species of farmed fish such as turbot and Atlantic cod (*Gadus morua*). Nevertheless, the production of vaccines against this virus is an area that has been little investigated.

In a previous study, we assayed oral DNA-based immunotherapy against IPNV [11] and the immune specific host reaction to a VP2-IPNV vaccine). The transcriptional changes produced by infection were determined in a rainbow trout 15k microarray designed by including annotated genes selected by key-words in the GenBank. However, since difficulties arose when trying to analize the results in terms of pathways, we tried to solve those problems by designing a user friendly and amenable programme to study this data, which is described below. We have illustrated the method with data obtained from this rainbow trout microarray to search for orthologous pathways in zebrafish, although the software can be applied to any other case or species of interest. Finally, the present work reports the search of some orthologous genes or proteins involved in several pathways from three teleost fish species (*Dicentrarchus labrax, Salmo salar and Oncorhynchus mykiss)* from KEGG database.

Materials and Methods

Database searches

We have used the following databases to design the programme's algorithm: (See 132 Supplementary File1) Nucleotide and Expressed Survey Sequence (EST): http://www.ncbi.nlm.nih.gov/nuccore/ and http://www.ncbi.nlm.nih.gov/nucest/): The Nucleotide Genome Survey Sequence (GSS) and Expressed Sequence Tag (EST) databases contain nucleic acid sequences typically uncharacterized such as short genomic (GSS) or cDNA (EST) sequences.

HomoloGene: (http://www.ncbi.nlm.nih.gov/homologene). It is a program that makes use of amino acid sequence searches (blastp) to find more distant relationships, although the procedure still refers to the DNA sequence to perform some of the statistics. Moreover, HomoloGene entries now include paralogues in addition to orthologues. Nevertheless, data for all species is still not available. For example, fish are only represented by zebrafish (*Danio rerio*).

Gene (http://www.ncbi.nlm.nih.gov/gene/): This database contains information on gene specificity, structure, function, homology between species and citations. The database supplies gene-specific connections in the nexus of a map, sequence, expression, structure, function, citation and homology data.

UniGene (http://www.ncbi.nlm.nih.gov/unigene/): This database groups transcript sequences from different loci based on genomic sequences. The availability of a genomic sequence is helpful to identify sets of transcript sequences that correspond to distinct transcript loci or to annotated genes.

Blast2GO (http://www.blast2go.com/b2ghome) is a programme to get homologous amino-acid sequences from nucleotide sequences. This research tool uses BLASTx to find the most similar sequence between several input sequences in a FASTA format.

KEGG (http://www.genome.ad.jp/kegg/) is a collection of manually drawn pathway maps, representing the molecular interaction and reaction networks for a number of cellular processes and genetic events. The database contains gene names and information on biological species-specific pathways. While searching the KEGG database with known pathways, we found that genes may be represented with several synonyms not all of which were linked to a pathway. Therefore, when the species of interest corresponding ID was not available in the KEGG database, it was first necessary to find the gene ID of the corresponding orthologous gene. Our programme provides this tool.

Microarray data

We previously studied the transcriptional changes induced by an oral DNA vaccine against the IPNV by using a rainbow trout microarray. Pooled RNA from the fish was hybridized on an Agilent rainbow trout microarray (8x15K format custom microarrays -ID032303-) containing 6442 60-mer oligo sequences. The annotation file of the microarrays was designed by us and might be provided by the supplier in ".txt" format (Ballesteros et al., 2012 for further details of the microarray used, the hybridization conditions and the first analysis). For the corresponding raw data see NCBI (http://www.ncbi.nlm.nih.gov/geo/), accession Number GSE31591. We used this microarray data as an example for the analysis and interpretation of the results from the Gene2Path programme.

Results

Software package for the analysis and interpretation of DNA microarray data

An scheme of the steps to follow the Gene2Path programme is shown in Figure 1, briefly the next steps are shown in Figures 2 and 3 (Supplementary File 2):

Dictionary, gene symbol and download of the FASTA data file

The dictionary section of the programme Gene2Path converts an input as accession numbers IDs into other code types available in the NCBI database (such as gene identification -GI- in GenBank).

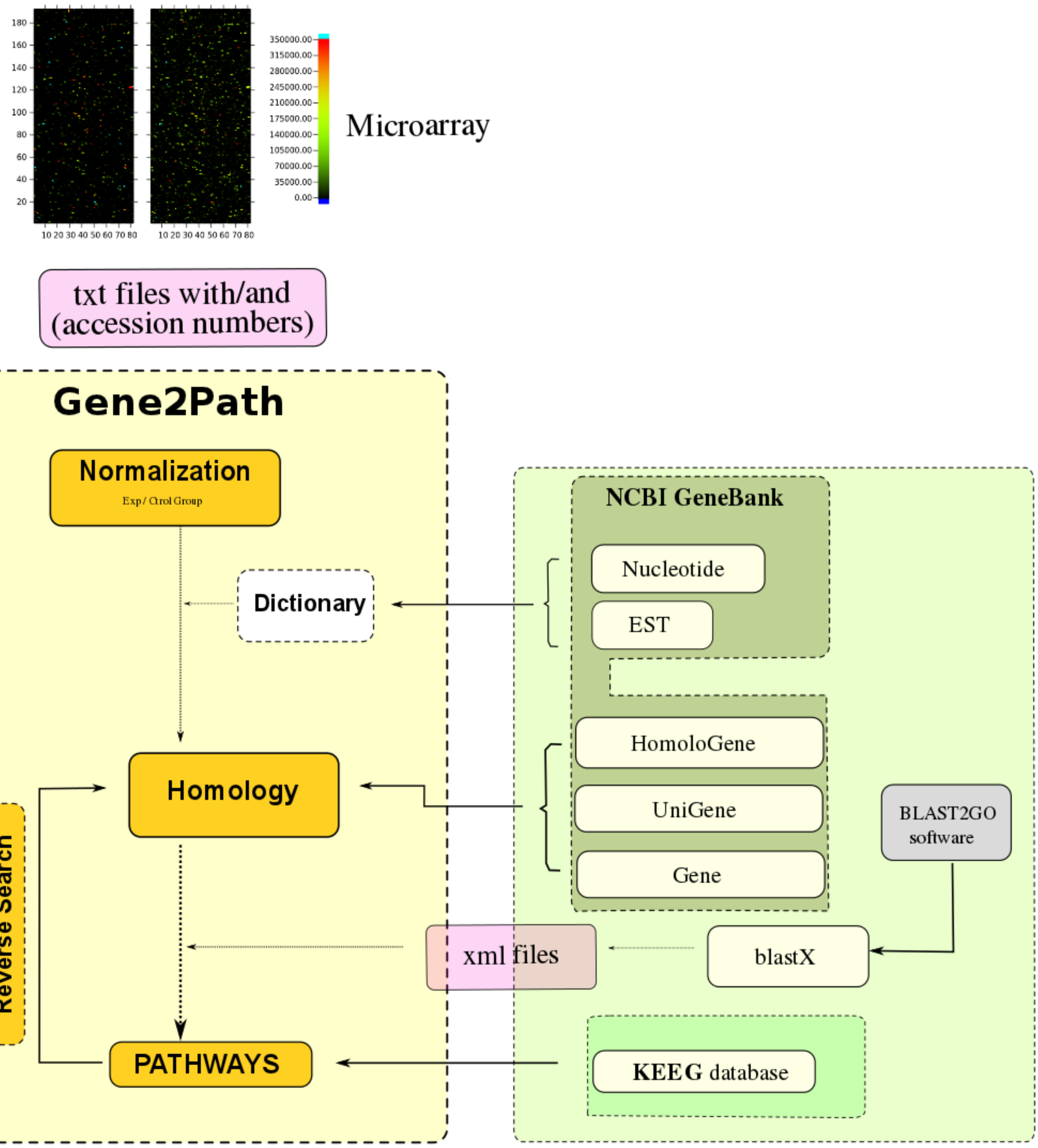


Figure 1: Scheme of the pathway analysis program and its search into different databases such as NCBI and KEGG. The diagram of Gene2Path is represented in the yellow cells, showing the automated steps of the program. The cells in green represent the databases or external programs used by Gene2Path. Arrows indicate the databases needed to follow up each one of the program steps. Into the rose cells are mentioned the archives extensions used by Gene2Path: txt are the gProccesedSignal of each of the genes from the microarray "input data"; the xml archives were obtained from the external program BLAST2GO and are used by the Gene2Path program to process and organize the blastX information. The Dictionary section provided, if needed, the gene identification codes (ID), obtained from the NCBI GeneBank for each one of the genes.

The scripts and programme are available at the "GENE2PATH" web site and they include the following features: (1) The addition of synonyms for gene identification by searching the NCBI database; (2) the analysis of the intensity of the gProccesedSignal reading of the synonym names microarray input files (extension txt); (3) Automated gene homology searches through the Homologene, Gene and Unigene NCBI databases; (4) An automatic filter was developed to find sequences similar by BLAST and to visualize the xml output file and to deal with several files at the same time; (5) Search for pathway information in the KEGG database using Gene IDs.

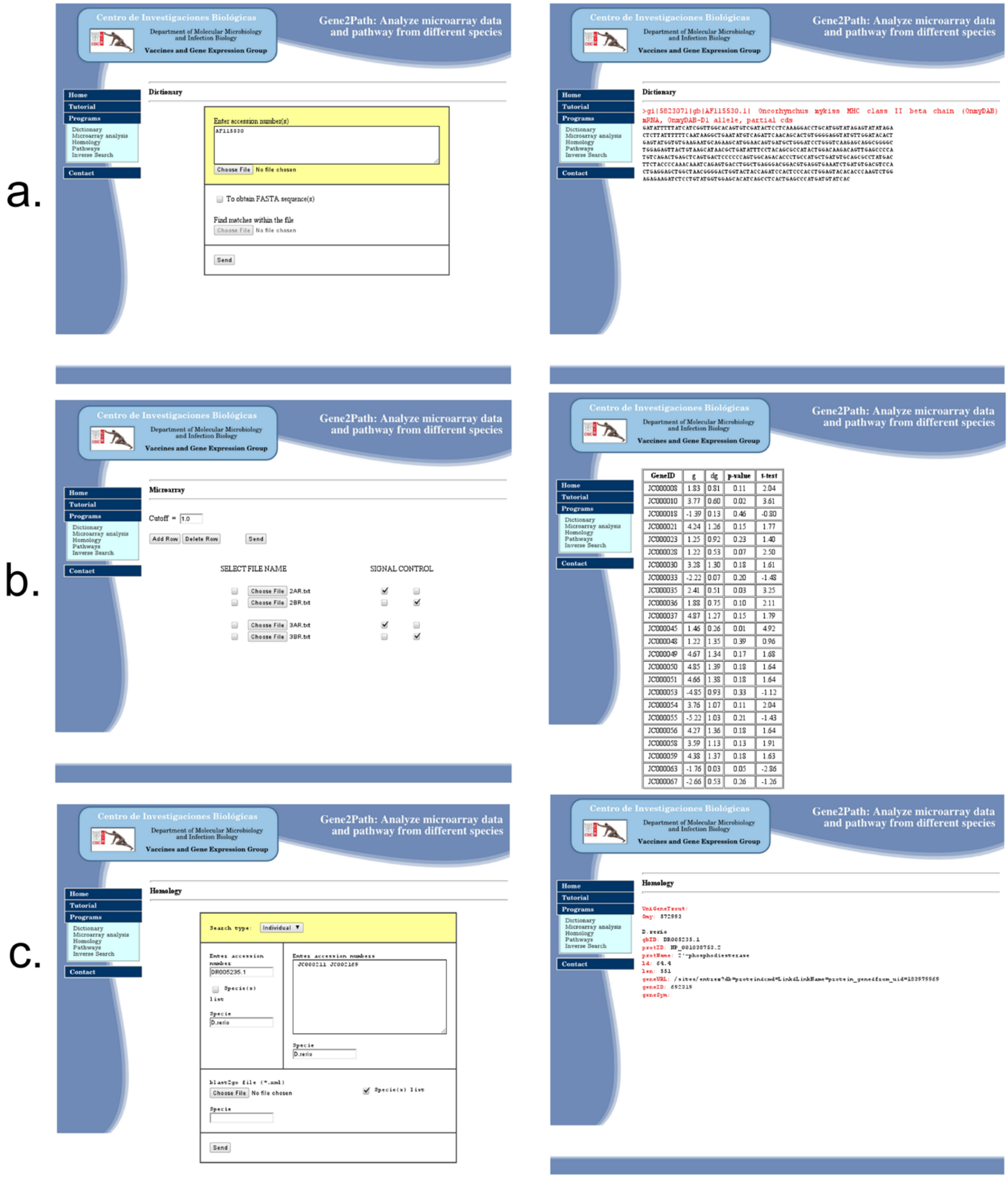


Figure 2: Illustrates the web tutorial of Gene2Path program. Successive images of the screens are shown **(a)** Dictionary step. **(b)** Normalization step. Values from the "g" signal process file in the ".txt" format were used. Data files correspond to control or experimental groups, and the gene expression fold with their t-test paired p-value. **(c)** Orthology step: Searching through NCBI.

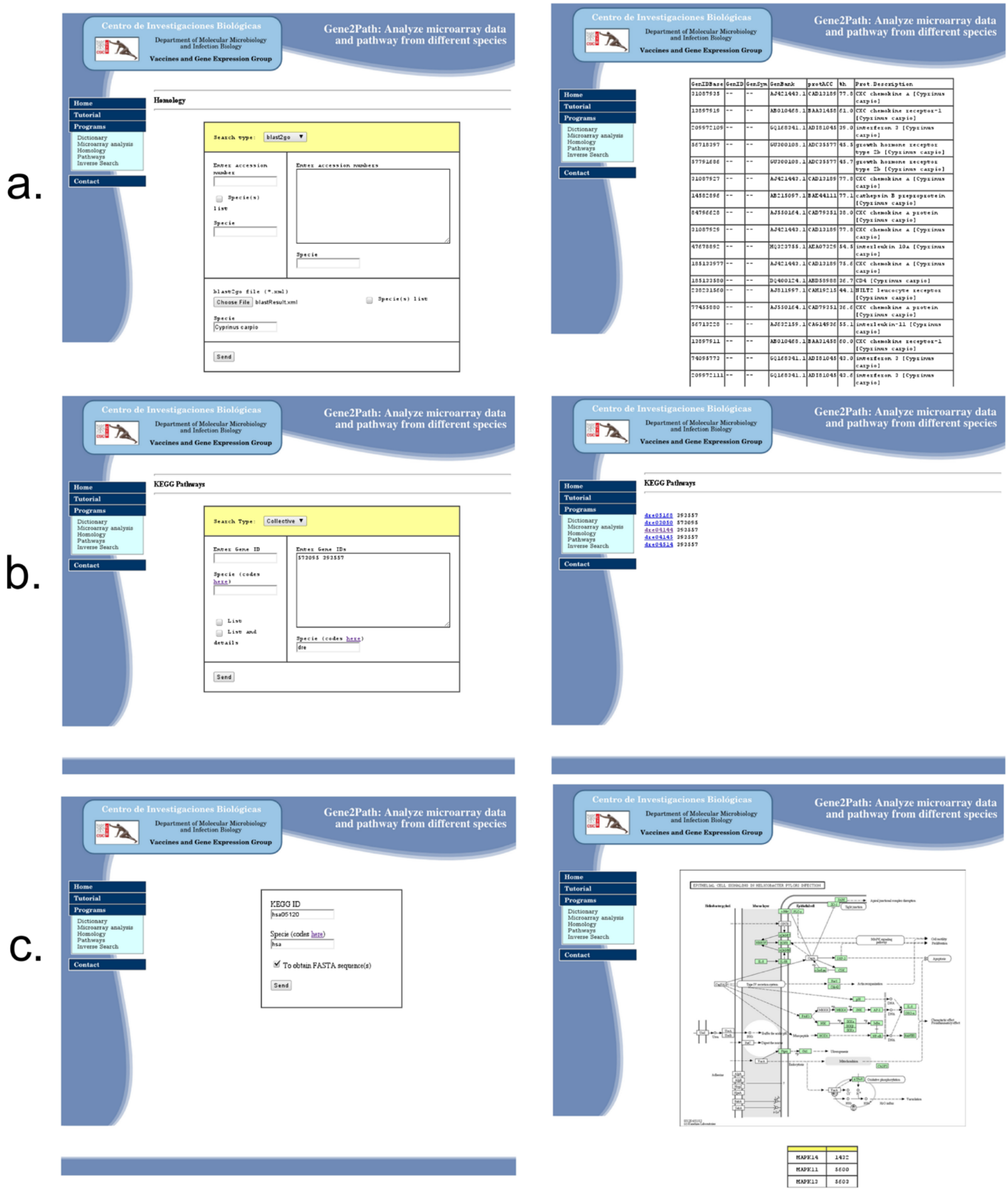


Figure 3: Illustrates the web tutorial of Gene2Path program. Successive images of the screens are shown **(a)** Orthology step: Searching through Blast2GO program. **(b)** Identifying pathways through KEGG. Gene IDs are necessary to search genes in the KEGG pathways involved. **(c)** Reverse Search of pathway. The web page shows the boxes or components separated by horizontal broken lines, which permits the genes to be assigned to a particular box.

Moreover, it is possible to obtain the corresponding gene sequences in FASTA format and to visualize the gene symbol (short name) for each of the genes in the microarray. The IDs given by the user are run through the Nucleotide or EST sequence database, named "nuccore" or "nucest", respectively (Figure 2a) and a final output list is provided.

Normalization and the level of gene expression from DNA microarray data

The normalization section of programme Gene2Path automates the analysis of the data generated by the microarray scanner. In our case example, values from the "g" signal process file in ".txt" format were used and most simple calculations were chosen (Supplementary File 3). Among the programme's options, the user can choose whether the data files correspond to control or experimental groups, and the level of gene expression to be visualized on the web site http://gene2path.no-ip.org/cgi-bin/index.cgi can be established. In our example, values ≥ 2 fold were selected. Finally, the program facilitates the process of data with t-test to obtain the p-value for statistics (Figure 2b).

Orthology

Identifying gene orthologous through NCBI

The programme allows the search of similarity of a deduced amino acid sequence from translated nucleotide to be determined between two species selected by the user. The procedure involves first searching for gene IDs in the GenBank web using Unigen, Gene and HomoloGene to detect orthology between the annotated genes or sequences of entire eukaryote genomes (Figure 2c). The results are visualized on the web and the percentage of homology is shown.

Identifying orthologous genes through BLAST2GO

Gene2Path filters and organizes the results from the Blast2GO program (".xml" files). Blast2GO uses the deduced amino acid sequence in order to find orthologous proteins in other species (blastx). As FASTA sequences from each of the genes are needed to run the Blast2GO programme, we can use data obtained in the Dictionary section (Figure 3a). The programme produces a table with information such as: The gene ID from the original or input species, gene ID in the orthologous selected by the user, gene symbol (short name) of the orthologous gene, GenBank entry of the gene sequence from the orthologous sequence, protein accession ID of the orthogous protein, percentage of homology and a short description of the gene (Figure 3a, right side).

Identifying KEGG pathways for the orthologous genes

The Gene2Path programme finds routes defined by the genes (pathways) in the KEGG database. Gene IDs are necessary to search for KEGG pathways containing those genes and gene IDs can be obtained from the orthologous genes, as described above. Each pathway is identified by its own ID (KEGG alias). To automate the search and the retrieval of the gene data from the KEGG database, a BASH script was written using the KEGG API. Direct links in each pathway were added to the file for each of the genes. This software can be used freely http://gene2path.no-ip.org/cgi-bin/index.cgi, with no need to register (Figure 3b).

Reverse Search of Pathway

The programme provides a tool to find genes involved in specific pathways by using the KEGG ID and recovering each of the genes shown in the pathway box through their gene IDs. The web page shows the boxes or components separated by horizontal broken lines, which permits the genes to be assigned to a particular box (Figure 3c). The user may obtain the sequences for each of the genes in FASTA format for the procedure. An example of a selected pathway and table, with the names of the boxes or KEGG components, as well as their corresponding Gene IDs is shown. This tool can be used to search for gene or protein orthologous involved in pathways because not all the pathways are available for a particular species in the KEGG data base. In this study, we selected potentially-relevant zebra fish and human (*Homo sapiens*, hsa and *Danio rerio*, dre respectively) pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.ad.jp/kegg/) for *Dicentrarchus labrax, Oncorhynchus mykiss and Salmo salar.*

The human ("hsa") and zebra fish ("dre") pathways were selected because they are the most complete and phylogenetically close, respectively, to our species interest. The KEGG pathways selected for study were: "Mapk signaling–dre04010; Apoptosis– dre04210; TGF-BETA signaling–dre04350; Toll-like receptor-dre04620; NOD-like receptor-dre04321; Cytosolic DNA-sensing-dre04623; Jak-stat signaling-dre04630; Herpes simple Infection-dre05168, Chemokine signaling-hsa04062; B-cell receptor-hsa04662; Fc-epsilon RI signaling-hsa04664; Bacterial invasión-hsa05100; Hepatitis C-hsa05160; Measles virus-hsa05162; Influenza A-hsa05164; HTLV-1-hsa05166 and NK-cell mediated-hsa04650". Some of these mammalian pathways have unknown fish equivalents. On the other hand, four of seventeen pathways were not found to *D. labrax* (see Supplementary File 4). The Mapk signalling pathway-dre04010 for all species is showed in Figure 4.

In summary, given one gene ID the user of Gene2Path program would be able to obtain which KEGG pathway is in. And given a pathway the user would got how many of the genes are in it.

Gene2Path analysis of the microarray gene expression results of rainbow trout genes following the administration of an oral fish DNA vaccine. A case study

To demonstrate the use of the Gene2Path, we used a dataset derived from an experiment previously reported**.** Thus, an oral alginate-microencapsulated DNA vaccine against VP2- IPNV protected in rainbow trout *Oncorhynchus mykiss* [11] against IPNV. The kidney and pyloric ceca from vaccinated and control fish, obtained 7 days post vaccination, were assayed using a microarray enriched in rainbow trout immune-related genes from the GeneBank and selected genes from a previous design. Our rainbow trout microarray (8x 15K), called minitrout 12.8 (Agilent ID032303), contains 6,442 unique 60-mer oligo sequences, each in duplicates arranged randomly in the microarray [11,12].

Data analysis procedure

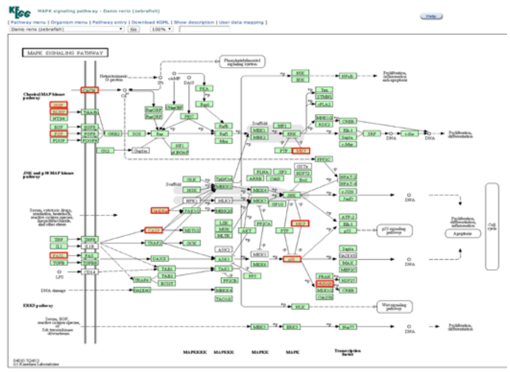
Step 1: Obtaining the rainbow trout gene IDs. Gene2Path was used to convert the rainbow trout mRNA accession number used for the microarray design into Gene IDs.

Step 2: Searching for orthologous genes in zebrafish. (Orthology step). The gene IDs or the accession numbers were used to search homoloGene, Gene and Unigene sections of the NCBI database, and BLASTx using BLAST2GO software for zebrafish (*Danio rerio*) orthologous. This step was obligatory to subsequently search for pathways in the KEGG database, as they are only available for some species. In this example from an input of 6442 rainbow trout accession numbers 1,282 orthologous IDs for zebrafish genes were found in the NCBI database.

D. labrax [1]

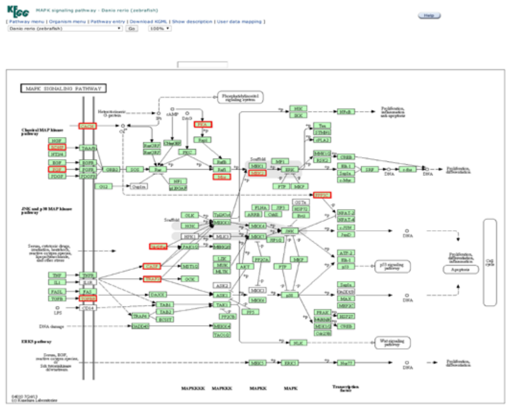
Gene ID NCBI/KEGG	Gene or protein ID species[1]	% orthology	Description gene or protein
100000665	CBN81685	73.2	Dual_specificity_protein_phosphatase_3_
58133	CBN80750	73.7	Nerve_growth_factor_
140621	CBN81450	51.5	Caspase_7,_apoptosis-related_cysteine_peptidase_
100002318	CBN80892	63.5	Mitogen-activated_protein_kinase_14a_
58118	ACN39714	81.6	brain-derived_neurotrophic_factor_
562552	CBN80893	59.0	Mitogen-activated_protein_kinase_14a_
557810	CBN80892	62.8	Mitogen-activated_protein_kinase_14a_
556226	NP_956926	77.9	cell_division_control_protein_42_homolog_isoform_1
100006208	CBN81227	65.1	Ras-related_C3_botulinum_toxin_substrate_1_
735295	AAZ20770	26.3	TNF-alphaTumor_necrosis_f...
266756	CBN80893	89.2	Mitogen-activated_protein_kinase_14a_
790934	CBN81049	78.7	MAP_kinase-activated_protein_kinase_3_
564273	CBN81378	93.0	Serine/threonine-protein_kinase_TAO2_
65237	CBN80893	88.6	Mitogen-activated_protein_kinase_14a_
30560	AAV90630	80.2	FGF6_

MAPK Signaling Pathway dre04010



O. mykiss [1]

Gene ID NCBI/KEGG	Gene or protein ID species[1]	% orthology	Description gene or protein
564295	NP_001117865	54.9	tumor_necrosis_factor_receptor_associated_factor_2
564337	NP_001154040	97.6	Calcineurin_subunit_B__>gi|225703570|gb|ACO07631.1
563181	NP_001117896	90.3	MAPK_/ERK_kinase__>gi|115345159|emb|CAI84980.2
140621	NP_001233264	65.5	casp3_protein__>gi|316979910|emb|CBY85134.1
795517	NP_001118156	61.1	v-mos_Moloney_murine_sarcoma_viral_oncogene-like
58118	ACY54685	83.2	BDNF,_partial_
567438	ABO87657	79.3	voltage-gated_calcium_channel_gamma-2_subunit_
556226	ACO08171	77.4	Cell_division_control_protein_42_homolog_
100006208	NP_001158622	65.1	Ras-related_C3_botulinum>gi|22570535...
792928	NP_001268297	86.1	TGF_receptor-1_>gi|386642816|emb|CBL93217.1|_...
30560	CAA76422	81.6	fibroblast_growth_factor_6-related_protein_
100538234	NP_001118061	94.0	testis_catalytic_subunit_of_cyclic_adenosine



S. salar [1]

Gene ID NCBI/KEGG	Gene or protein ID species[1]	% orthology	Description gene or protein
65239	NP_001117181	90.8	mitogen-activated_protein_kinase_gi|55509092
568951	NP_001135384	86.2	Dual_specificity_mitogen-activated_protein_kinase
564337	ACN58626	99.4	Calcineurin_subunit_B_isoform_1_
563181	NP_001133281	91.1	Dual_specificity_mitogen-activated_protein_kinase
556443	NP_001158853	57.4	adapter_molecule_crk;gi|223647708|gb|ACN10612.1
140621	AAY28975	57.1	caspase_7_
557926	NP_001133568	88.5	Serine/threonine-protein_phosphatase_2B
100002318	ABL68016	63.5	p38b1_mitogen_activated_protein_kinase_
562552	3OHT_A	58.0	Chain_A,_Crystal_Structure_P38alpha
557810	ABL68016	60.9	p38b1_mitogen_activated_protein_kinase_
777735	NP_001158799	76.2	fibroblast_growth_factor_12;gi|223647478|gb|ACN10497.1
563727	NP_001167256	73.9	Mitogen-activated_protein_kinase_kinase8_gi|223648904|...
556226	ACI33560	71.6	Cell_division_control_protein_42_homolog_precursor_
100006208	ACI66728	63.6	Ras-related_C3_botulinum_toxin_substrate_2;gi|209735...
100000342	NP_001158821	69.1	Microtubule-associated_gi|223647564|gb|ACN10540.1
735295	NP_001134790	41.1	Tumor_necrosis_factor_ligand_superfamily;gi|209736050...
792928	ACN11406	65.7	Activin_receptor_type-1B_precursor_
567833	NP_001158821	43.6	Microtubule-protein_tau_gi|223647564|gb|ACN10540.1|...
266756	ABL68016	88.5	p38b1_mitogen_activated_protein_kinase_
790934	ACI33697	72.1	MAP_kinase-activated_protein_kinase_2_
100538181	ACN58636	68.3	Mitogen-activated_protein_kinase_kinase_kinase_kinase_2_
65237	ABL68016	87.0	p38b1_mitogen_activated_protein_kinase_
678610	ACN58636	68.3	Mitogen-activated_protein_kinase_kinase_kinase_kinase_2_
561006	ABO13872	66.6	DAXX__>gi|148362139|gb|ABQ59664.1|_DAXX_
559222	ACN11406	85.9	Activin_receptor_type-1B_precursor_
100538234	ACN10609	94.0	cAMP-dependent_protein_kinase_catalytic_subunit_beta_
100002272	NP_001133901	57.4	Dual_specificity_protein_phosphatase_22-Agi|209155756|

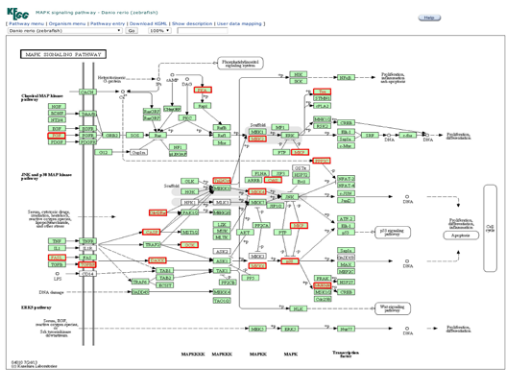


Figure 4: Results obtained after running the Reverse Search step of Gene2Path program in the MAPK signalling pathway dre04010 of three fish species. Headings are: the gene ID from *D. rerio* (first column), the gene ID of the fish species under study (second column), the percentage orthology (third column) and a short description of the gene or protein of the species selected (fourth column). The figure (right side) illustrates the situation of orthologes genes into the pathway.

Step 3: Search zebrafish pathways with orthologous genes: It is interesting to note that only 12 genes into 10 different pathways were detected in KEGGS by using the ID gene symbols provided by the microarray. This search was run without the orthology step (original data https://earray.chem.agilent.com/earray/search.do?search1/4arrayDesign); however, after running the Gene2Path program (accession data 2013-01-15), the numbers increased to 1169 genes and 179 pathways (Figure 5). The identification of 169 additional pathways with the Gene2Path programme demonstrates its efficacy. Although genes may be active in more than one pathway. The microarray used was designed to determine transcriptional changes in selected immune genes induced by a DNA vaccine and hence, identification of immune related pathways is to be expected. Nevertheless several other pathways were also detected, such as those involved in "apoptosis and the cell cycle" (23 different KEGG pathways), "regulation of energy metabolism" (42 different KEGG pathways), and several pathways that could be grouped without direct network associations. Figure 6 illustrates the data of the intestinal immune network for IgA production (KEGG alias: dre04672).

Only two genes were obtained manually from the KEGG database, without using the "orthology step" are represented in Figure 6a. The same pathway is shown after running the "orthology step" on the Gene2Path software (Figure 6b); from the 55 genes of the pathway, 18 genes were detected, which represents a 33% increase. IgA production pathway has not been yet identified in fish, but functional similarities with the IgT genes implicated in the *Danio rerio* orthologous could exist, and pathways showed similar trends than most of those pathways described.

Moreover if a user needs to know the specific genes involved in a pathway, or if there is interest in focusing on a specific section of the pathway, it is possible to obtain these genes through the "Reverse Search of pathways" application of Gene2Path. The results of the pathway analysis of the microarray data provide insight into differential organ-specific biological processes that may explain the differences in host response to the VP2-IPNV vaccine.

Discussion

In this article, we describe a pathway-based approach to analyze microarray data from uncommon biological species using newly designed Gene2Path software. To achieve this, we relied on the orthology of the genes identified in a microarray with those from other species included in the KEGG database. The programme automatically searches pathway databases to analyse microarray data in an independent, species-specific way. (For installation, instructions, examples and source code see Supplementary File 5). We have illustrated the method with data obtained from a rainbow trout microarray to search for orthologous pathways in other well known biological species, such as zebrafish, although the software can be applied to any other case or species of interest. Large scale gene expression studies represent an important advance in experimental molecular biology. Microarrays have become an important tool in functional genomics studies and they are often used to address a variety of biological situations but in some cases they are structured to well know biological species [3]. Such studies rapidly generate large quantities of gene expression data, the handling of which represents a major challenge for biologists. Indeed, the importance of presenting microarray data in the framework of documented biological pathways has often been noted [4,5]. In biology, pathway is a set of interactions or functional relationships between the physical and genetic components of a cell that operate in concert to fulfil a biological requirement. The databases that capture information on these functional interactions of molecular species are numerous, and the lack of uniformity of models and the methods to access this data makes integrating pathway data extremely difficult for uncommon biological species. Thus most of the software packages use species-specific gene IDs and they cannot handle gene data from other species. Yet it is necessary to make such pathway information systems more flexible and efficient, since while data for humans and common laboratory animals such as mice are widely available in databases through the internet, this is not so for other species such as those economically important fish species. The zebrafish (*Danio rerio*) is a model organism for genomic studies and a variety of functional pathways from this species can be found in the databases. The same is true for human databases, the most studied species at the genomic level. These pathways can be used as models to integrate and visualize data from microarray experiments from other species (rainbow trout in our case).

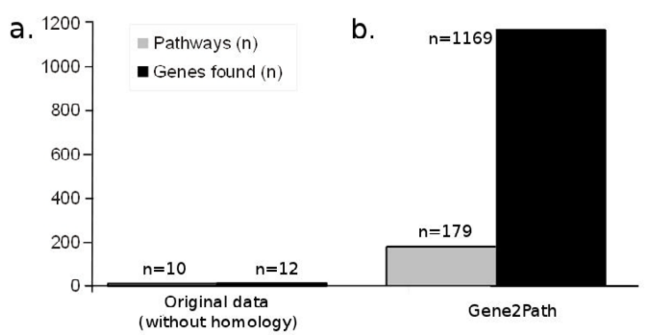


Figure 5: Number of pathways and genes found by using the gene IDs from a rainbow trout microarray (original data of the microarray species) and numbers obtained after running the Gene2Path software. The reference orthologous species was *Danio rerio*. The program provides the gene IDs in this species for searching pathways into KEGG database.

The microarray pathway analysis tool described here can be applied to a typical experiment in which two conditions are compared to identify genes whose differential expression changes significantly with respect to the reference condition. We used a microarray to analyse the differential expression of immune-related genes induced by the administration of an oral DNA vaccine in two rainbow trout organs, a species that is much less well represented than zebrafish in the pathway databases. The principal motivation for building pathway databases is to make tools available that help answer specific biological questions. The majority of genes in most genomes have no known function and examining genes in the context of a particular pathway may help to elucidate their role. For example in our case, a gene of unknown function connected to a set of genes involved in early immunity is likely to also act in this process. However, the power of many pathway analysis techniques is proportional to the amount of input data. Rainbow trout-specific pathways are not available and therefore, we have used pathway data from other species (such as humans or zebrafish), for comparision. Pathway analysis software tools, such as STARNET 2 [13], Reactome database [14] and CYTOSCAPE [15], are available, although again they are only applicable to humans and some experimental animals (mouse and rat). On the other hand, Babelomic (http://babelomics.bioinfo.cipf.es), GEPAS (http://www.gepas.org), are a set of free programs for the analysis of data from microarrays [16]. These softwares are very comprehensive and useful; however, it is necessary to work with human, mouse or other species, because the programs lack an orthologous step beyond the most usual

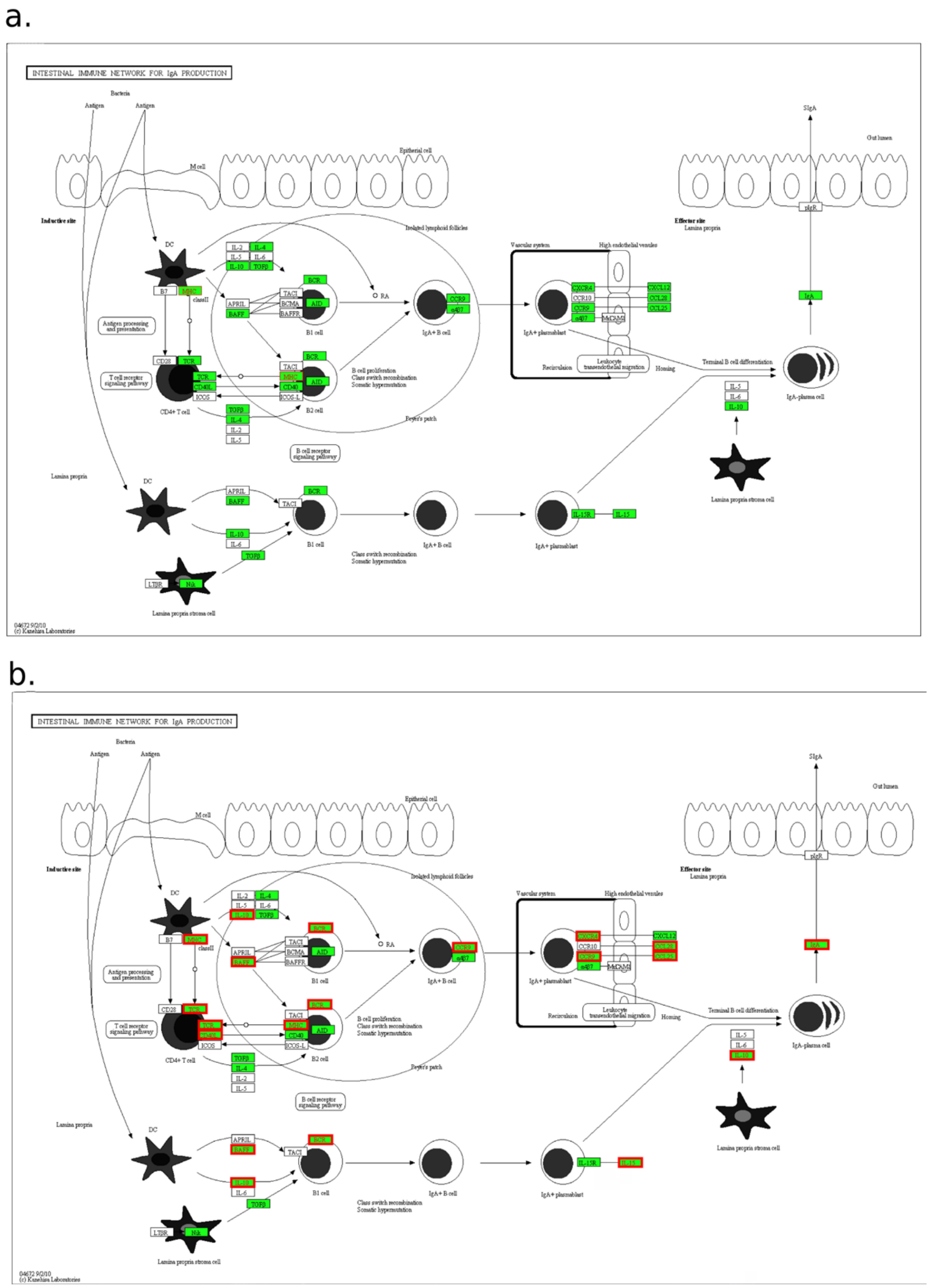


Figure 6: An example showing the results obtained in a pathway when processed or not by Gene2Path: Illustration of the "Intestinal immune network for IgA Production" using the data from a rainbow trout microarray. (A) Genes (red boxes) found in the *Danio rerio* pathway (KEGG alias: dre04672) obtained manually from the databases (n=2/55 genes), without the orthology step. (B) Genes found (red boxes) in the same pathway after running the orthology step of the program (n=20/55 genes). IgA have not been identified in fish, but functional similarities with the IgT genes implicated in the *Danio rerio* orthologous could exist and pathways showed similar trends than most of those pathways described.

biological species. Our program offers the possibility to perform orthology searches in other biological species such as fish. The software described here, and the application to one example, show how results from microarray experiments can be integrated into pathways and visualized by using one "gene orthology" step even with uncommon biological species. This enables to drawn pathways in species which are not supported in the KEGG database. The issue here was to derive knowledge of biological relevant patterns in genetic profiling data that were related to the teleost's immune defences. Accordingly, the role of several genes revealed by the pathway comparison could be defined. Another advantage of the automated procedure used by the Gene2Path software is that no direct supervision is needed and once the process has been initiated, the user can leave the programme running and visualize the results later. In the Agronomic, soils and environmental sciences department have been developed some user friendly software that can be used in industrial companies related with healthy, safety, environment (HSE). Some examples of that software are: Environmental flow diagram (EFD) [17], Soil Heat Calculator Program (SHCP) [18], and Optimize the infiltration parameters in Furrow irrigation using Visual Basic and genetic algorithm [19]. In summary the Gene2Path programme performs an automated search of several databases over 5 steps. (1) The addition of synonyms to identify genes by searching the NCBI database by their IDs. (2) The analysis of the intensity of the gProccesedSignal reading in the one-channel microarray output files (extension ".txt"); (3) An automated search of gene orthology through the homologene, Gene and Unigene NCBI databases, whereby the tool compares nucleotide sequences and comparative 3D models of proteins (constructing an atomic-resolution model of the "*target*" protein from its amino acid sequence, and producing an experimental 3D structure of a related orthologous protein). (4) The identification of sequences similar to the query set in NCBI nr and EST databases using Xblast. Since other programmes now exist for this step, such as BLAST2GO, we developed an automatic filter to readily visualize the output file (".xml) that enables several files to be analyzed at the same time; (5) A search of KEGG database pathway information using orthologous genes (Gene IDs).

Conclusion

All the software steps were applied to the microarray data we had obtained previously from vaccinated fish: The software proved to be very efficient in terms of automation and data processing. For instance, running a search of different NCBI databases renders 2/3 genes per second (depending on the internet connection speed). The analysis of the data from the vaccinated fish in our example rendered 179 targeted pathways. The Gene2Path software allows the automated searching of NCBI databases and the straightforward visualization of the data retrieved based on a graphic network environment.

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2. Optimizar los métodos de administración de vacunas DNA orales.

Sistemas de administración de vacunas DNA en acuicultura:

En peces, la vía de administración de vacuna más utilizada es im. o parenteral. Recientemente, se ha considerado la vía oral, por lo cual se han desarrollado diferentes sistemas para incorporar vacunas en microesferas (Kanellos et al. 1999). Es esencial que el pDNA encapsulado este correctamente enrollado para su eficiente transfección y posterior traducción (J. Tian, Yu, et al. 2008). Además es necesario evitar su degradación en el intestino.

2.1. Un método efectivo para la administración de vacunas a través del pienso

En trabajos previos demostramos que la vacuna de DNA "pcDNA-VP2" frente al IPNV, administrada a los alevines de trucha por vía oral de forma manual e individualmente , es capaz de estimular al sistema inmune innato y adquirido de la trucha y protege a los peces de la infección por IPNV (RPS del 80-82%). Con objeto de optimizar la vacuna y proporcionar un método más fácil de administración que genere menos manipulación al pez y así disminuir su estrés, se incorporó la vacuna directamente al pienso de los peces. Para lo cual, se añadieron las micropartículas recubiertas con alginato liofilizadas al pienso.

Diseño experimental:

Alevines de trucha arco iris de aproximadamente, 1.5 g, 3.5 - 4 cm de longitud

- Grupo de peces (n=25) alimentados con pienso comercial suplementado con pcDNA-VP2 recubierta con alginato de sodio durante 3 días, la dosis fue de [10 µg] por pez por día A los 15 días post vacunación, se infectan los peces con 3 X 10^5 $TCID_{50}$/ml de IPNV Sp durante 2 horas por inmersión.
- Grupo de peces (n=25) alimentados con pienso comercial suplementado con pcDNA recubierto con alginato de sodio "PV" durante 3 días, la dosis fue de [10 µg] por pez al día. A los 15 días post vacunación, se infectan peces con 3 X 10^5 $TCID_{50}$/ml de IPNV Sp durante 2 horas por inmersión
- Grupo de peces (n=25) infectados con IPNV Control infectados. Se alimentan con pienso comercial sin tratamiento durante 15 días. Luego, se infectan con 3 × 10^5 $TCID_{50}$/ml de IPNV Sp. durante 2 horas por inmersión
- Grupo de peces control (n=25), sin tratamiento.

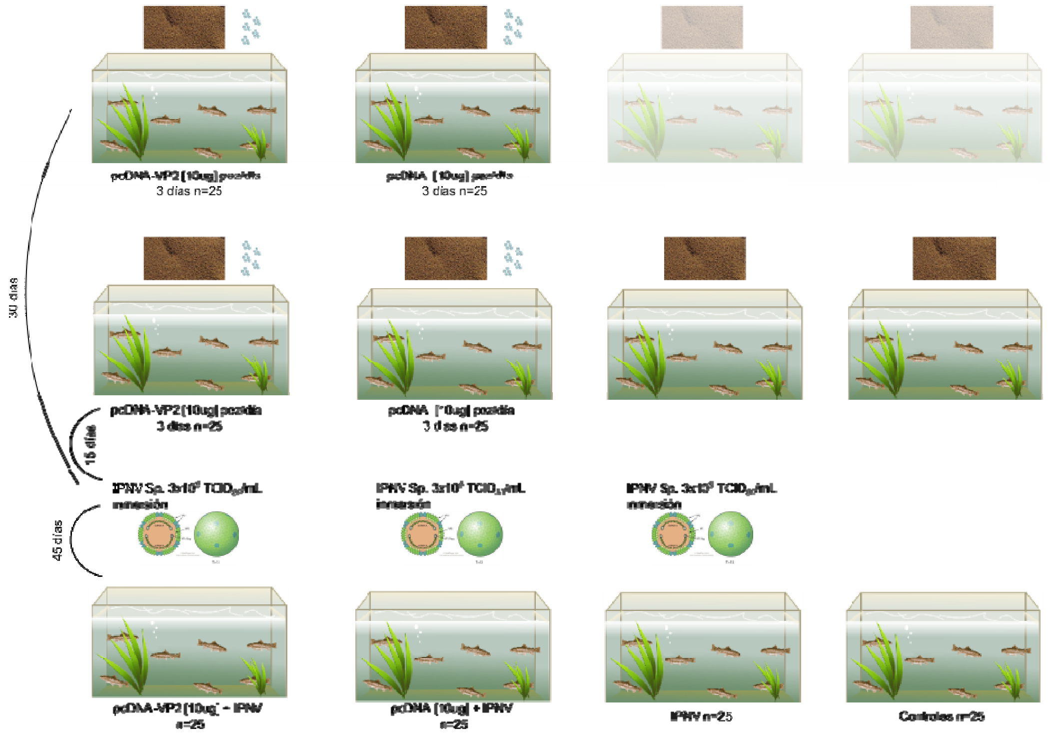

Figura 1: Esquema experimental

Tiempos de muestreo: 1, 3, 5, 7, 15 y 30 días después del tercer día de tratamiento. En peces infectados se les extrajeron vísceras a los 15, 30 y 45 p.i.

Muestras: Riñón anterior, Bazo, Branquias, Intestino Posterior.

Métodos: Extracción de RNA total utilizando TriZol, síntesis de cDNA, PCR cuantitativa a tiempo real.

Resumen:

La expresión del gen VP2 (gen insertado) de la vacuna DNA pcDNA-VP2 se detectó en varios órganos de truchas alimentadas con el pienso mezclado con la vacuna ("medicado"). Además, se demostró que la vacuna administrada mezclada con el pienso comercial era capaz de inducir tanto una respuesta inmune innata (expresión de genes IFN-I, IFN-γ, Mx1, IL8 e IL12), como específica (expresión de genes IgM, IgT, CD4 y CD8), y una clara protección ante la infección viral, con tasas de supervivencia relativa del 78% al 85,9%. También se comprobó la producción de niveles detectables de anticuerpos neutralizantes al menos durante los 90 días, de toma de muestras. Podemos concluir que la experiencia de la vacuna en el alimento fue un éxito puesto que la ingesta fue homogénea y el nivel de protección logrado más alto incluso que vacunando individualmente con pipeta y la vacuna en solución. No obstante, para determinar la posible influencia del material de recubrimiento en la optimización de la vacuna, se ensayaron otros compuestos de encapsulación.

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Full length article

Food pellets as an effective delivery method for a DNA vaccine against infectious pancreatic necrosis virus in rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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A B S T R A C T

A DNA vaccine based on the VP2 gene of infectious pancreatic necrosis virus (IPNV) was incorporated into feed to evaluate the effectiveness of this oral delivery method in rainbow trout. Lyophilized alginate–plasmid complexes were added to feed dissolved in water and the mixture was then lyophilized again. We compared rainbow trout that were fed for 3 consecutive days with vaccine pellets with fish that received the empty plasmid or a commercial pellet. VP2 gene expression could be detected in tissues of different organs in the rainbow trout that received the pcDNA-VP2 coated feed (kidney, spleen, gut and gill) throughout the 15 day time-course of the experiments. This pcDNA-VP2 vaccine clearly induced an innate and specific immune-response, significantly up-regulating IFN-1, IFN-γ, Mx-1, IL8, IL12, IgM and IgT expression. Strong protection, with relative survival rates of 78%–85.9% were recorded in the vaccinated trout, which produced detectable levels of anti-IPNV neutralizing antibodies during 90 days at least. Indeed, IPNV replication was significantly down-regulated in the vaccinated fish 45 days pi.

1. Introduction

The control of infectious diseases is essential to maintain the levels of productivity in the aquaculture industry. The economic impact of infectious diseases, particularly those of viral aetiology, is a constant worldwide threat in the salmonid fish industry, stimulating research to find efficient methods to minimize such losses. Vaccination is the most effective approach to combat disease in aquaculture, a strategy that is ideal to prevent and avoid the dispersion of infective viruses in fish, particularly in farms where fish are raised under intensive culture conditions. Although different types of viral vaccines have been described for fish, including inactivated, attenuated, synthetic peptides or subunit vaccines [1–3], protection is not always complete. Hence, studies are necessary to produce improved vaccines capable of inducing longer lasting immunity and less stressful methods of administration [4]. Genetic vaccines were first developed for mammals in the 1990s and several designs to protect against rhabdoviruses have been tested in salmonid fish species [5–12]. More recently, other DNA vaccines have been described to combat the infectious pancreatic necrosis virus (IPNV), another viral pathogen of salmonid fish [13–15].

Infectious pancreatic necrosis virus (IPNV) is the type species of the Aquabirnavirus genus, from the *Birnaviridae* family [16]. Virions are non-enveloped and they contain two segments (A, B) of double-stranded RNA. Segment A is the larger of the two (about 3.1 kbp) and it encodes VP2 and VP3, the two major structural proteins of the virus [17,18]. The VP2 protein is the type-specific antigen that can induce the production of neutralizing antibodies that are capable of protecting susceptible fish from viral infection [19–21]. IPNV is one of the main causes of mortality worldwide for juvenile salmonid fish, being especially destructive in salmonid eggs and fingerlings [22]. Fish surviving IPN epizootics develop a persistent viral infection or carrier state, capable of continually transmitting the virus to other susceptible populations of fish, including their own offspring [23–26] (for reviews see Refs. [22,27,28]).

Genetic vaccination for IPNV has only recently been undertaken experimentally, and the initial steps in its development have focused on traditional injection methods [13]. Intramuscular injection of DNA vaccines has been successfully used against viruses such as infectious haematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV). Intraperitoneal injection has also been routinely used for other vaccines, such as recombinant vaccines and multivalent products, and automated systems for

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1050-4648

injection have been developed [3]. However, oral vaccines would be easier to administer and they represent an important alternative to immunize fish against viruses. Nevertheless, there are still few reports of successful vaccine delivery methods other than injection, with delivery in feed having been mostly licensed to prevent bacterial but not viral diseases [4].

We previously described a DNA vaccine derived from the VP2 gene of IPNV inserted into an expression plasmid and encapsulated into alginate microspheres. The oral delivery of the plasmid (diluted in PBS) was performed manually in order to ensure the uniform vaccination of the fish under study. Strong protection was achieved in this way, with around 83% relative survival when challenged 15 and 30 days after vaccine delivery. Indeed, strong expression of IFN and the IFN-induced antiviral Mx protein was recorded 7 and 15 days post-vaccination (pv) [14,29]. However, novel approaches to improve the efficacy of DNA vaccine oral delivery would not only provide interesting data regarding the future mass delivery of these vaccines but also, keys to understand the cellular and mucosal immunity reactions. Oral delivery of DNA vaccines is a process that has been poorly explored, especially against IPNV. Thus, having generated a vaccine that successfully induces appropriate immune protective responses, the next step should be to check if this vaccine can be delivered in feed without losing its beneficial effects due to the severe conditions experienced during gastrointestinal transit.

The goal of the present work was to determine the effectiveness against IPNV of the pcDNA-VP2-encapsulated in alginate and incorporated into fish feed, and the immune responses it induces in rainbow trout. Given that this method appears to produce similar results to those described previously, its potential should be further assessed for industrial application.

2. Materials and methods

2.1. Ethics statement

The experiments described comply with the Guidelines of the European Union Council http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm (Directive 2010/63/EU) for the protection of animals used for scientific purposes and were previously approved by the CSIC Ethics committee.

2.2. Cells and virus

The BF-2 cell line from bluegill fry (*Lepomis macrochirus*, ATCC-CCL 91) was used to isolate and propagate the viruses. The IPNV Sp strain from the ATCC was used in this study (ATCC VR 1318), and all the cells and viruses were cultured as described previously [30]. Briefly, cells were grown at 25 °C in Leibovitz's medium (L15, Gibco, Spain) supplemented with 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine and 10% foetal bovine serum (FBS: Gibco, Spain), or with 2% FBS in the maintenance medium (MM). The virus was cultured in L-15 medium and propagated at 20 °C. The virus was titrated in 96-well culture plates (Falcon, Becton–Dickinson) infected with 10-fold serial dilutions and the plates were observed daily for the development of a cytopathic effect (CPE). The infective titres were determined as the 50% infective dose in tissue culture ($TCID_{50}$ mL^{-1}): based on Reed & Muench [31].

2.3. Fish

Rainbow trout (3.5–4 cm and 1.5 g mean size and weight) were purchased from a local spring water farm with no history of viral disease. The fish were kept at the "Centro de Investigaciones Biológicas" (CSIC, Madrid, Spain) under a 12/12 h light/dark regime at 15 °C in 350 L closed re-circulating water tanks (Living Stream, Frigid Units Inc, Ohio). The fish were fed daily with a diet of commercial pellets and they were maintained as described elsewhere [32]. To assess their health, pools of five fish were examined for viruses by standard protocols [33,34], none of the fish lots examined giving positive results. The trout were anaesthetized with buffered tricaine methanesulphonate (MS-222, Sigma) prior to handling and the experiments described comply with the European Union Guidelines (86/609/EU) for the use of laboratory animals.

2.4. Oral vaccination

2.4.1. Vaccine and preparation of fish feed

The pcDNA-VP2 plasmid in *Escherichia coli* (TOP10) was prepared as described previously [14]. Cultures were grown in 10 L of LB broth and the cells were then recovered by centrifugation and frozen at −20 °C. The plasmid was purified from the cells using the QIAGEN plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. The pcDNA-VP2 and pcDNA plasmids were coated with sodium alginate and these microspheres were prepared as described previously [29]. The feed (T-2.0 Nutra from Trow España S.A, Burgos, Spain) was that recommended for fish that are approximately 3.5 cm long and that weigh 1.5 g, and it was the same as that used at the farm that provided the rainbow trout. This feed contains 54% protein from fish-meal, 18% oil, 1% cellulose, 11.5% ash, 3% calcium, 1.2% sodium and 1.7% phosphorus, as well as several other oligo-elements and anti-oxidants. The particle size ranged from 1.0 to 1.7 mm.

In our previous studies with vaccine–alginate complexes, the fish received daily drops containing 10 μg vaccine. In the present work, the size and weight of the vaccinated trout were similar (3.5 cm, 1.5 g) and since the fish are thought to ingest around 5% of their body weight daily, each fish should receive 0.075 g of feed/day. Thus, the experimental vaccine was prepared in lots of feed for 120 fry trout by placing commercial dry pellets (27 g) into 50 ml Falcon tubes and along with 3.6 mg of pcDNA-VP2-alginate microspheres previously diluted in 15 ml of distilled water. The pellets and microspheres were then mixed gently for a few minutes at room temperature, and the middle-moist feed obtained was lyophilized for 24–48 h and conserved at 4 °C until it was used. In this way, a vaccine concentration of 10 μg per fish and day was achieved.

2.4.2. Fish vaccination

Groups of 25 trout were placed in separate 40 L aquaria maintained at a constant temperature of 15 °C for treatment. The first group of fish was vaccinated by providing with vaccine impregnated food pellets at 5% of body weight for three consecutive days (10 μg pcDNA-VP2/fish/day). The second group of rainbow trout was fed with pellets mixed with the empty pcDNA plasmid, serving as the plasmid control, and the third group of fish received the commercial pellets and was considered as the untreated mock vaccinated fish control. An additional group of fish were vaccinated individually with a pcDNA-VP2 alginate microspheres solution in water, and used as a positive control in the light of the results obtained with this method elsewhere [29].

2.4.3. Tissue distribution and time-course of pcDNA-VP2 expression

At 1, 3, 5 and 15 days pv (after the last feed), 3 trout from each group were sacrificed with an overdose of MS-222, and the kidney, spleen, liver, gut and gill tissue was removed aseptically. Total RNA was isolated using the TRIzol reagent (Invitrogen, Spain), according to the manufacturer's instructions, and treated with DNase I to remove any trace genomic DNA that might interfere with the PCR reactions. Equal amounts of RNA were primed with oligo(dT) and cDNAs were synthesized to analyse the VP2 gene expression by real-time PCR as described previously [14,34,35].

2.4.4. Gene expression

At days 1, 2, 3, 5 and 7 pv, trout were sacrificed ($n = 3$) and the head kidneys were processed for RNA extraction. We analysed the expression of five genes by RT-qPCR, three belonging to interferon-related pathways (IFN-1 [36], IFNg [37] and Mx-1 [38–40] and two interleukins IL8 [41] and IL12 [42]). On days 15 and 30 pv, the kidney of trout ($n = 3$) from each group was also processed to evaluate the expression of genes related to adaptive immune responses, such as IgM [43] and IgT [44]. All the primers used had been reported and optimized in previous studies [34,35]. The transcription of CD4 and CD8 Th cell markers was also assessed using the primers CCTGCTCATCCACAGCCTAT (F) and CTTCTCCTGGCTGTCTGACC (R) for CD4 and AGTCGTGCAAAGTGG-GAAAG (F) and GGTTGCAATGGCATACAGTC (R) for CD8. The corresponding accession numbers are AY973030.1 and NM_001124263, respectively. All qPCR reactions were performed in triplicate and for each mRNA, and gene expression was normalized to that of the endogenous control (elongation factor 1-α; EF1-α) and expressed as $2-^{\Delta Ct}$, where ΔCt was determined by subtracting the average EF1-α Ct value from the average target Ct. The change in expression relative to the empty plasmid pcDNA was also determined for some of the samples by applying the formula $2^{-\Delta\Delta Ct}$ [45] where $\Delta\Delta Ct = \Delta Ct$ of samples of target gene $-\Delta$Ct of the calibrator (pcDNA control).

2.5. Titration of neutralizing antibodies

A neutralization test (NT) was used to evaluate the specific immune response against IPNV in the vaccinated trout. Fish blood was collected by caudal puncture at 15, 30, 60, and 90 days pv, and the IPNV neutralizing antibody titre was analysed in the serum of individual fish. This assay involved incubating a two-fold dilution of the serum with a known amount of reference IPNV, and the mixtures were then assayed in triplicate on BF-2 cells. Similarly, IHNV was also tested to assess the specificity of the reaction. After incubating for 3 days, the cell cultures were fixed and stained with a crystal violet solution (2% in ethanol). The titre of a serum was determined as the reciprocal of the serum dilution that reduced viral infectivity by approximately 50% when compared to the virus control ($TCID_{50}$ mL^{-1}). Titres >40 were considered positive.

2.6. IPNV challenge

To examine the efficacy of the DNA vaccine, the vaccinated and control groups of fish were challenged with IPNV and monitored for cumulative mortality. Rainbow trout were divided into three groups (25 fish each) and were vaccinated by feeding with pellets containing the encapsulated pcDNA-VP2 plasmid on 3 consecutive days (10 μg DNA/fish/day: group 1). A second group 2 received similar amounts of pellets impregnated with the encapsulated empty pcDNA plasmid and the third group of fish was fed with commercial pellets. An additional group of fish were vaccinated individually with a pcDNA.VP2 alginate microspheres solution in water, and used as a positive control in the light of the results obtained with this method elsewhere. Water quality was maintained at optimum levels and all tanks were kept under equivalent conditions. On day 15 or 30 pv, each group of fish were challenged with IPNV by immersion (1×10^6 $TCID_{50}$ mL^{-1}), as described previously [29]. Fish mortality in each group was subsequently recorded and any dead fish were removed daily over the next 30 days.

Vaccine efficacy was determined by comparing the average cumulative percentage of mortality (cpm) and the relative

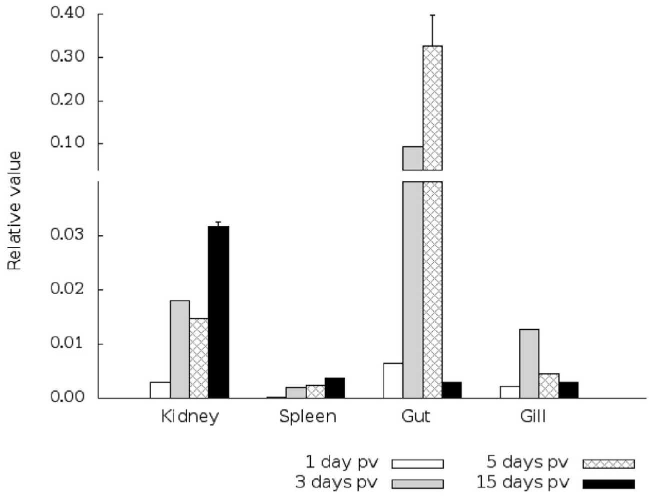


Fig. 1. Relative quantification of VP2 gene expression assessed by real-time PCR. Total mRNA was obtained from different tissues (head kidney, spleen, gut, and gill) at 1, 3, 5 and 15 days post-vaccination with pcDNA-VP2. The vaccine was incorporated into commercial pellets and the fish were fed the DNA vaccine diet on three consecutive days. The first day without feeding vaccine pellets was considered day 1 post-vaccination. The data are shown as the mean gene expression relative to the expression of the endogenous control EF-1α ($2^{-\Delta Ct}$ method). The error bars represent the St. dev. of the mean ($n = 3$).

percentage of survival (RPS: [1 − (% mortality in vaccinated fish/% mortality in control fish) × 100]). Two replicas of the trial were carried out.

2.7. Expression of the IPNV-VP4 gene in fish after vaccination and IPNV challenge. Isolation of virus from survivors

The expression of the IPNV-VP4 gene was used as a measure of the replication of IPNV in the control fish, and in the vaccinated and infected fish. Three trout from each group were sacrificed with an overdose of MS-222 on day 45 post-infection (pi) and their kidney tissue was removed aseptically. Total RNA was isolated and cDNAs were synthesized to analyse IPNV-VP4 gene expression by real-time PCR (see above). The VP4 gene serves as a marker of IPNV, making it possible to distinguish the VP2 vaccine expression from the IPN viral expression. The relative level of infection in the vaccinated and infected fish was then estimated by RT-qPCR analysis.

The virus was isolated by processing head kidney samples from the same fish assayed by RT-qPCR, and BF-2 cells were inoculated with aliquots of the homogenates as described previously [32].

2.8. Statistical analysis

Prior to performing statistical analyses, the normal distribution of the data was checked and confirmed using the Shapiro–Wilk test. The data are presented as the mean ± standard deviation (St. dev.) of the results from three trout. An analysis of variance (factorial ANOVAs) was run to determine whether the differentially expressed gene differed between the replicates for each individual gene, followed by Tukey's multiple comparison test to assess the differences between the vaccinated and control group. The Student *t*-test was also used to compare some paired samples. All statistics were analysed with the IBM SPSS Statistics package, an integrated family of products that addresses the entire analytical process, from planning and data collection to analysis, reporting and deployment

(IBM® SPSS® Statistics 15; http://www.spss.com). $P \leq 0.05$ was considered significant.

3. Results

3.1. Tissue distribution of the VP2 gene

To assess whether the DNA vaccine delivery approach tested may be suitable for immunization against IPNV, the tissue distribution of the VP2 gene was first studied. The presence of mRNA-VP2 was quantified in several organs over time and in the kidney of the vaccinated fish, and VP2 gene expression increased strongly from 3 days to at least 15 days pv. When compared to the fish tested on day 1, relative increases in expression of around 4.7 fold and 12.5 fold were detected at 3 and 15 days pv, respectively (Fig. 1). The spleen was the organ where the weakest relative expression of VP2 was recorded at any of the time points selected. The strongest expression of VP2 was recorded in the gut with an increase at 3 days and with a peak of expression on the 5th day, when the relative expression was around 52-fold that on day 1. However VP2 expression in the gut decreased dramatically to basal levels on the 15th day. This VP2 transcript was also detected in the gill, although again at lower levels.

3.2. Transcriptional changes of immune-related genes in the kidney of rainbow trout 7 days post-vaccination

We previously used a specific microarray to analyse the genes expressed by rainbow trout 7 days pv with the pcDNA-VP2 [34]. Some of the genes found to be differentially expressed in the vaccinated fish, and/or induced during IPNV infection of trout, were also studied here. These expression profiles were evaluated to demonstrate that oral delivery of the plasmid and VP2 expression induced innate and adaptive responses.

Administration of pcDNA-VP2 in the feed induced a strong increase in the expression of the genes selected to reflect an innate-immune response to the virus. The increase in expression of the marker genes induced in vaccinated fish is shown relative to the fish that received pellets containing the empty plasmid (pcDNA: Fig. 2). The increase in expression induced by the pcDNA-VP2 vaccine was strongest for IFN-1 and IL12, with values ranging from 57 to 82-fold, while the expression of the Mx-1 and IL-8 was also up-regulated on day 3 pv (25- and 47-fold, respectively). The expression of all the genes tested was significantly up-regulated for several days, with the highest increases observed on the second to the third day pv. The significant up-regulation of IL-12 gene expression persisted throughout the experiment and it was approximately 13-fold at 7 days pv. By contrast, the mRNA expression of all the other genes decreased to near basal levels at 7 days pv.

3.3. Transcriptional changes in several adaptive immune-related genes in the kidney of rainbow trout at 15 and 30 days post-vaccination

Of the many genes involved in the specific immune-response we choose to study: i) the IgM gene that is related to cellular immune responses; ii) IgT, recently associated with mucosal immunology; and iii) the expression of the CD4 and CD8 genes, T-cell markers, considered to play an important role in eliminating virus-infected cells. The changes in gene expression in vaccinated fish relative to the pcDNA group of fish were assessed (Fig. 3) and the strong IgM and IgT expression detected in the kidneys 15 days pv increased significantly at 30 days pv (around 17- and 11-fold, respectively). By contrast, appreciable levels of CD4 and CD8 genes were recorded but no significant changes were observed at the times assayed.

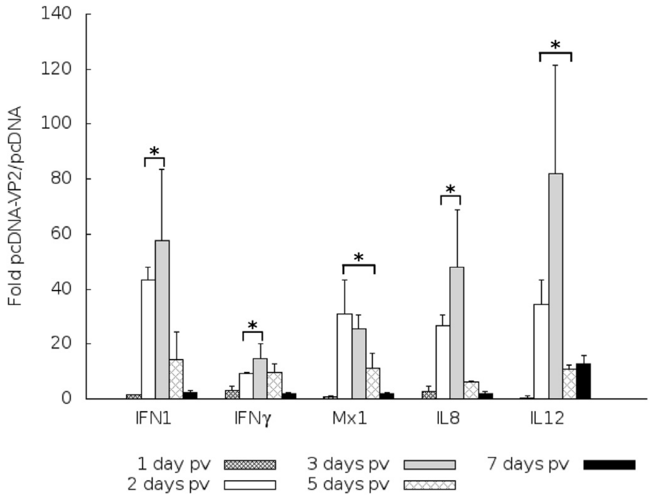


Fig. 2. The change in expression of immune innate-related genes assayed by RT-qPCR in the rainbow trout kidney at 1, 2, 3, 5 and 7 days post-vaccination with pcDNA-VP2 plasmid incorporated into the diet. The vaccine was administered on three consecutive days and the first day without feeding vaccine pellets was considered day 1 post-vaccination. The head kidney tissue from 3 fish was collected and the RNA extracted for RT-qPCR analysis, which was performed in triplicate. The endogenous EF-1α gene was used to normalize the results and the data are presented as the mean fold increase relative to fish fed with the pcDNA empty plasmid pellets (±standard error: $2^{-\Delta\Delta Ct}$ method). Asterisks indicate significant differences ($P \leq 0.05$) relative to the data from day 1.

3.4. Neutralizing antibodies

The anti-IPNV neutralizing antibodies were examined in serum samples from fish at 15, 30, 60 and 90 days pv (Table 1). Anti-IPNV

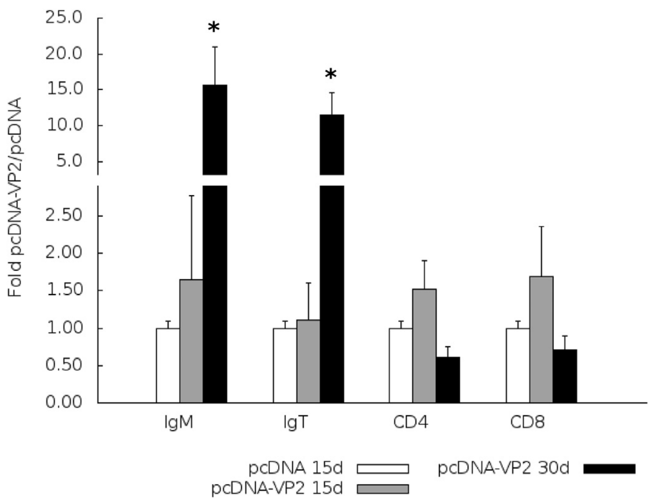


Fig. 3. The change in expression of IgM, IgT, CD4 and CD8, adaptive-related immune genes, assayed by RT-qPCR in rainbow trout kidney 15 and 30 days after dietary pcDNA-VP2 vaccination. The vaccine was administered on 3 consecutive days and the first day without feeding vaccine pellets was considered day 1 post-vaccination. A second group of fish received the food pellets impregnated with the empty pcDNA plasmid. Total mRNA was obtained from the head kidney and the fold increase in transcripts compared to the empty plasmid controls was assessed. The error bars represent the St. dev. of the mean ($n = 3$) and the asterisks indicate statistically significant differences relative to the control at 15 days pv ($P \leq 0.05$).

neutralizing antibodies were detected in 18 of 20 fish in the group fed with pcDNA-VP2 and tested at 15 and 30 days pv, whereas all the fish tested at 60 and 80 days had anti-IPNV.VP2 antibodies. Neutralizing titres were higher in these late groups, from 160 to 640. No antibodies against IPNV were detected in the sera from the control fish (saline solution administered) and fish that received the empty plasmid pcDNA. In these studies a titre ≥40 was defined as positive and low titres of anti-IPNV neutralizing antibody were only observed in 3 of the 40 total vaccinated fish.

The specificity of the induced antibody was demonstrated by the failure to neutralize IHNV. A progressive increase in the anti-IPNV neutralizing antibodies was recorded, demonstrating the antigenic potential of the VP2 gene.

3.5. *Cumulative mortality and relative percent survival (RPS) after IPNV challenge to vaccinated fish*

When rainbow trout were analysed 15 or 30 days pv and challenged with IPNV (Fig. 4), a cumulative mortality of 83% was observed in the challenged control fish (A: IPNV control), as well as in the fish fed pellets containing the empty pcDNA plasmid (B). When the kinetics of mortality were analysed in this later group, it was slightly retarded with respect to the virus control group until the 20th day post-challenge when the mortality rates were similar. By contrast, the fish vaccinated via the feed were strongly protected, with a relative percent survival (RPS) of around 86%. Similarly and as expected, there was strong protection of fish that received the vaccine individually as a microspheres emulsion delivered by pipette (RPS = 73–76%), in which the onset of mortality was also delayed when they were challenged at 30 day pv. However, in this particular group the cumulative mortality profile was the highest of all the groups vaccinated in these experiments. The fish vaccinated individually or through the feed, and that were challenged on the 15th day pv, exhibited parallel mortality curves from 15 to 25 days post challenge. Likewise, similar proportions of these fish died, and they also resisted the challenge better than the corresponding groups challenged at 30 days pv. Nevertheless, at the end of the experiment the strongest protection was induced in the fish administered the vaccine with the feed and that were challenged at 15 or 30 days pv (85.9% and 78.2% RPS, respectively), reflecting the efficiency of vaccination by this route of administration.

3.6. *Expression of the VP4 gene from IPNV at 45 days post-challenge and the isolation of the virus from survivors*

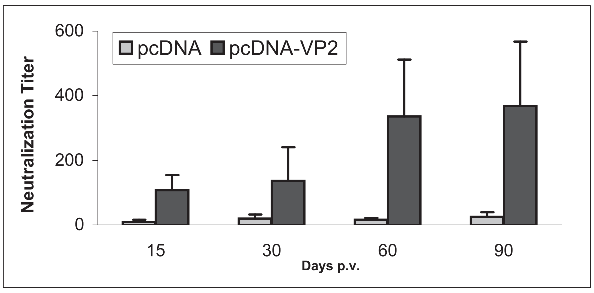
Viral gene transcription that involved the VP4 gene was analysed by real-time PCR using RNA from the head kidney as the template to evaluate viral load. The expression of the gene encoding the IPNV-VP4 protein was determined by RT-qPCR in the fish that survived 45 days post-challenge, those infected control fish group ($n = 3$), as well as in the vaccinated and challenged fish ($n = 3$). The VP4 gene was examined so as to distinguish between vaccinated and infected trout (Fig. 5), and its expression in the virus control group was 7-fold greater than in the vaccinated and infected fish in which there was a significant reduction in expression. These results suggest that IPNV replication was significantly down-regulated or that very low levels of the virus were found in the vaccinated fish 45 days pi.

4. Discussion

The present study set out to determine if addition of an IPNV-DNA vaccine to food pellets could serve as an efficient method of vaccine delivery, inducing an immune response and protecting rainbow trout against IPNV infection. Accordingly, we demonstrated that this vaccine was taken up by the fish and that the plasmid passed across the gut barrier, allowing the transcript to be efficiently expressed. The time-course experiments over 15 days confirmed the expression of the antigenic gene in all the organs examined. Among these, vaccine expression was strongest in the kidney for at least the first 15 days pv, while the expression in the gut peaked at 5 days pv but decreased to basal levels at 15 days pv. This profile could be explained by the abundant presence of the

Table 1
Neutralizing antibody titres in sera samples from fish immunized with the vaccine or the pcDNA plasmid in the feed.

Samples	Days post-vaccination			
	15	30	60	90
pcDNA-VP2[a]	80	320	320	160
	160	80	160	640
	80	40	320	160
	160	80	640	640
	80	80	640	320
	160	320	320	160
	160	40	160	640
	80	80	320	320
	40	160	160	320
	80	160	320	320
	<20	<20	<20	<20
pcDNA-VP2[b]	<20	<20	<20	<20
	<20	<20	<20	<20
	20	40	20	40
	0	20	20	10
pcDNA[a]	10	5	20	20
	10	20	10	20
	5	10	10	40
Saline solution[a,b]	0	0	0	0



pcDNA-VP2: Sera from fish fed with plasmid that encodes the IPNV VP2 gene; pcDNA: Sera from fish fed with empty plasmid.
Different fish were examined from 15 to 90 days post-vaccination. The antibody titre is the reciprocal of the highest dilution of the antiserum protecting against the standardized virus.
The histograms show mean values of neutralization titres against IPNV. Bars represent standard deviation.
[a] Sera tested against IPNV.
[b] Sera tested against IHNV.

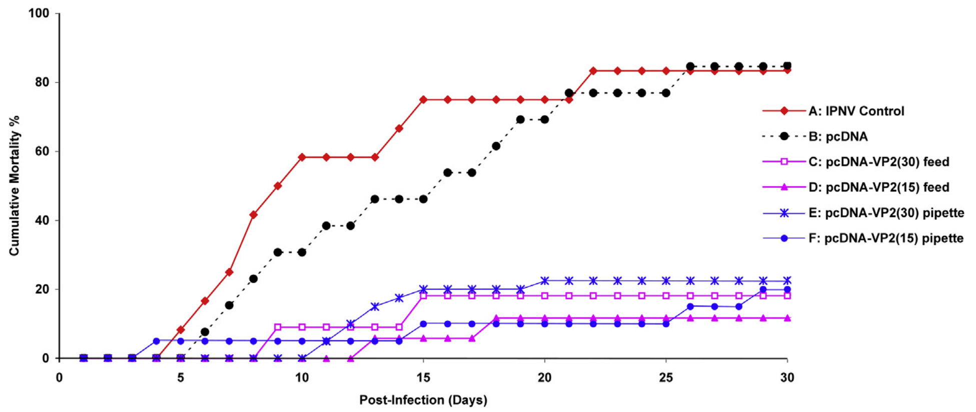


Fig. 4. Cumulative mortality of rainbow trout (1.5 g mean weight) challenged by immersion with 5×10^5 $TCID_{50}$ mL^{-1} of IPNV. The fish ($n = 20$ in duplicate tanks) were (A) mock-vaccinated with commercial food pellets and infected with IPNV, (B) mock-vaccinated with the empty pcDNA plasmid and infected with IPNV; (C, D) fed pellets incorporating the pcDNA-VP2 vaccine on 3 consecutive days and infected with IPNV; (E, F) pcDNA-VP2 vaccine individually administered by pipette tip. The fish were challenged on post-vaccination day 30 (C and E) and 15 (D and F), and monitored for up to 30 days.

plasmid soon after administration and the progressive passage across the intestinal barrier into the bloodstream, as suggested for other vaccines [46].

The expression of some representative genes was assayed to determine whether the vaccine was eliciting an immune response.

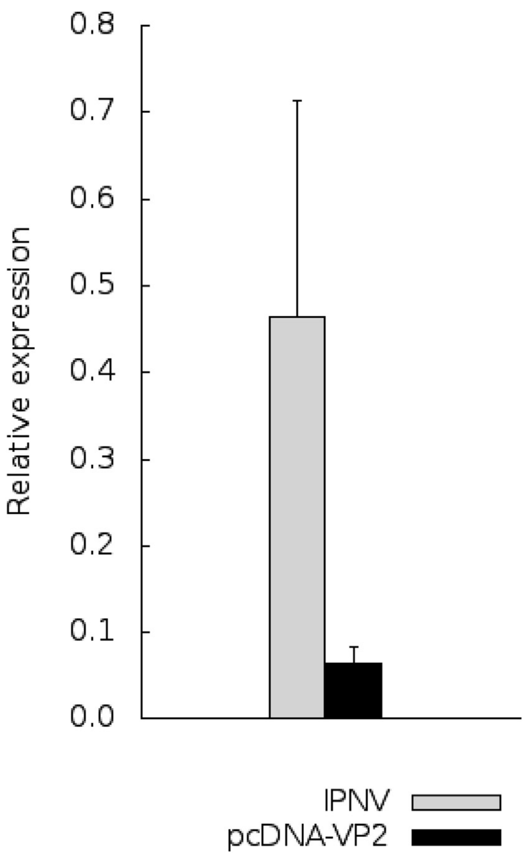


Fig. 5. Relative expression of the IPNV-VP4 gene in the IPNV infected fish, and in the vaccinated and challenged fish group 45 days post-challenge. The fish were vaccinated with pcDNA-VP2 through the diet on 3 consecutive days and they were then challenged with the virus (5×10^5 $TCID_{50}$ mL^{-1}) after 30 days. A second group was mock-vaccinated and infected with IPNV (virus control group). Transcription of the VP4 gene was recorded in the kidneys of both vaccinated and virus control fish at 45 days post-challenge, and the data are represented relative to EF1-α expression ($2^{-\Delta Ct}$ method), (mean + S.E., $n = 3$). Values with asterisks are significantly different ($P \leq 0.05$).

The transcriptional profile of genes involved in the immune response in the trout head kidney was determined previously by microarray hybridization after oral administration of the vaccine (plasmid–alginate complexes in water, administered individually through a pipette tip) [34]. From the large number of the genes up-regulated, fourteen of those related to immune responses were selected for quantitative RT-qPCR validation. In the present work, several IFN and interleukin-related genes, as well as the IgM and IgT genes, from that study were selected to analyse the time-course of their expression in the head kidney. These genes were considered to be suitable markers to assess the immunization procedure related to both the innate and specific responses. Other immune markers, such as those of the CD4 and CD8 T-cell lineage, have only recently been characterized in salmonids and their expression was determined here.

Among the non-specific immune events, it is well established that fish can secrete type I interferon in response to viral infection and it is one of the gene markers used to studying the early protection induced by a vaccine. Our results clearly show that the pcDNA-VP2 vaccine up-regulates IFN-1 expression, with early increases of around 57-fold with respect to the empty plasmid. A comparison with previous results with the alginate coated vaccine [29] suggests that the expression of the innate-immune-related genes was clearly higher in the fish fed with pcDNA-VP2 vaccine pellets. As expected, IFN-1 was expressed most strongly 2–3 days pv, although significant up-regulation of IFN-γ was also evident after 5 days (also named IFN-II), as also observed at different times for Mx-1, IL8 and IL12 in the vaccinated fish with respect to the fish fed with the empty plasmid. In turn, fish IFN II can induce the expression of many interferon induced genes (ISGs) that also respond to type I IFNs, suggesting cross-activation of the innate antiviral responses elicited by type I and II IFNs. Our results showed that the VP2 vaccine also modulates pro-inflammatory cytokines like IL-12, which was significantly up-regulated at 2, and even 7 days pv. Moreover, interleukin 8 (IL-8) was also rapidly up-regulated, a gene included in the CXCa group of chemokines, a family of cytokines that regulate immune cell migration under both inflammatory and normal physiological conditions. These genes not only promote leukocyte mobilization but also, they regulate the immune responses and differentiation of the recruited cells. Accordingly, they have been catalogued as key regulators of the immune response, acting as a bridge between the innate and adaptive responses [47]. Strong IL8 expression has been also reported following IHNV and VHSV infection, suggesting a role for this cytokine in viral defence [48,49].

We also evaluated the expression of genes representative of the adaptive immune response, immunoglobulins (Ig) M and T, and the Th cell markers CD4 and CD8. In mammals, Igs can be divided into five categories and for a long time, it was believed that only one of them, IgM, existed in fish. However several other functional Igs have recently been discovered in teleost fish and Ig genes were identified in rainbow trout, named IgT, similar to zebra fish IgZ [50]. It was also recently reported that rainbow trout contained a new B linage uniquely expressing surface IgT [44]. It is interesting that genes related to adaptive immunity were already being expressed by the 7th day after the last feed with the vaccine (the 10th day after the first feed), which could be relevant for the effectiveness of the vaccine.

We recently studied immune responses in the rainbow trout gut and pyloric caeca region, the area in which a major recruitment of B cells was demonstrated [51]. Moreover, we found significant increases in the number of both IgM^+ and IgT^+ intraepithelial lymphocytes after manual oral vaccination with the pcDNA-VP2 vaccine. In the present work, the head kidney was the target organ assayed, considered to be the primary lymphoid tissue in teleost fish and thus, an important source of B cells. The IgM and IgT genes were evaluated here at 15 and 30 days pv when the adaptive immune responses may be more apparent. Interestingly, the expression of both these genes peaked 30 days pv, suggesting the presence of Ig secreting cells, and corroborating the vaccine activity and the gene response to oral stimulation.

The level of expression was also evaluated for other genes, such as CD4 and CD8, although the transcription of these T cell genes was not significantly enhanced. This might be explained by differences between fishes and their ingestion ability, as well as the times selected for the assay. IFN-γ is mainly produced by $CD4^+$ cells in mammals and this gene was significantly up-regulated at 7 days pv, suggesting an activation induced by the vaccine. Further sampling of other organs and assessing the time-course of expression will be necessary to study the mechanisms and pathways that an oral vaccine could initiate in more depth. Here, we only aimed to assay the viability of the feed-delivery method to induce a variety of immune responses. Nevertheless, we demonstrated that VP2 is expressed in organs and that it orchestrates immune responses.

A detailed comparison with the results we obtained previously with an oral vaccine cannot be carried out, since the vaccine–alginate complexes were administered individually on only one dose [34,35] while here the fish were fed with the vaccine over 3 consecutive days. Nevertheless, similar profiles of expression were observed over the first 3–7 days using both methods of oral administration, although the response was stronger when the vaccine was administered in the feed. Peaks of expression were recorded around the 7th day post-vaccination with a pipette, and on the 3rd day (more-or-less) in the present study, which is 6 days after the fish received their first feed with the vaccine. With regards IgM and IgT expression, data were only recorded after the first 8 days post-vaccination in the previous study, when there was only weak induction of these genes ($\leq$3-fold). Here these antibody responses were examined at 15 and 30 days post-vaccination, considered as markers of adaptive immune response, when values around 15-fold were recorded. With regards protection, both methods to deliver the pcDNA.VP2 vaccine diminished the mortality from the very first days post-challenge, which may be related to the innate-immune response induced. Moreover, strong specific protection was evident 30 days post-challenge, as demonstrated by the levels of the neutralizing antibodies recorded and by the cumulative mortality. Taken together, these data show that the pcDNA.VP2 vaccine administered with the feed is stable in the gastric system of the fish, allowing efficient absorption of the antigen, as well as the subsequent induction of innate and adaptive responses.

To fully assess the effectiveness of the vaccine and delivery method, vaccinated and control fish were challenged with virulent IPNV at 15 and 30 day pv, and cumulative mortality was examined over 30 days. The vaccinated trout displayed strong protection, similar levels to those previously described for fish that were individually vaccinated with alginate-encapsulated plasmid [29]. The cumulative mortality was similar in the replicate experiments performed, despite the possible variability in feed uptake over the 3 days of vaccination. Interestingly, very low levels of mortality were recorded during the first 10–12 days in all the vaccinated fish, suggesting an antiviral effect induced by the activation of IFN and other genes involved in immune responses. Mild protection was also initially evident in the pcDNA vaccinated fish, although their final mortality was the same as in the virus control group. In the vaccinated fish the early protective effect seems to be followed by a later phase of specific immunity, as the vaccine induced the production of neutralizing antibodies against IPNV. Since the survival rates recorded are higher when fish were challenged on the 15th day pv, an overlap of both the innate and adaptive protective effects might occur at this time. Despite the role of non-specific defence mechanisms, it is noteworthy how effective the oral DNA vaccine and the VP2 antigen are in progressively inducing the appearance of neutralizing antibodies, and hence, specific protection.

Viral load was quantified by RT-qPCR at 45 days post-challenge in vaccinated virus challenged fish and in virus control trout. Fish from both groups were survivors that displayed no clinical symptoms of disease, yet the expression of the IPNV-VP4 gene detected in the infected non-vaccinated fish was significantly higher than that recorded in those that received the vaccine, suggesting that vaccination reduced viral load. However, PCR detection of a viral gene does not necessarily imply the presence of infective virions, as demonstrated by failure to recover IPNV after inoculation of cell cultures with homogenates from the head kidney from fish (data not shown). Experiments over longer periods than those carried out here would be necessary to determine whether PCR could detect the virus after several months. Meanwhile our results indicate that in the present conditions, the oral vaccine clearly diminished the viral load in the fish. Thus, these results demonstrated that administration through feeding is a promising delivery method, at least for IPNV vaccines.

Few studies on DNA oral vaccines against this or other fish viruses have been performed to date. Some preliminary experiments with an oral vaccine against IHNV did not prove to be as efficient (unpublished data) and a recent study on oral administration of a DNA anti-IHNV vaccine described inefficient protection, although the viability of oral delivery of DNA vaccines was demonstrated [52].

Protection is likely to increase as the doses, encapsulating materials and methods to add the vaccine to the feed improves. The effective uptake of the DNA vaccine and its transcription by enterocytes along the five segments of the digestive tract was suggested in our recent study in which the vaccine was delivered manually as microspheres suspended in solution [51]. The pyloric caeca was the area of the digestive tract in which a major recruitment of B cells was detected and the most responsive to the oral IPNV vaccination of the five segments examined. In this work, the increase in VP2 mRNA transcripts at 3 days rose further to peak in the gut after 5 days of vaccination. The second segment of the gut (as described by Rombout et al. [53]) is considered the region where a strong uptake of macromolecules takes place after the fish were fed for 3 days. However, while VP2 expression peaked in the kidney at 15 days pv, only basal levels of expression were recorded in the gut at that time of vaccination, suggesting that plasmid DNA was then redistributed to other tissues and only small amounts of VP2 expression persisted at this site.

The pattern of VP2 expression was different in the gut and kidney, peaking at 5 and 15 days pv, respectively, and producing stronger expression in the intestine. This is interesting because our knowledge of mucosal immunity is progressing and the recent discoveries regarding gut immunoglobulin open up new opportunities for the study of oral vaccines. It is believed that the teleost fish intestine does not contain organized lymphoid tissue but rather, the equivalent of M-cells that can capture, transport and present antigens to the underlying mucosal immune system [53,54]. The distal intestine of salmonids is well known to have strong vesicular transport activity compared to the proximal intestine [55]. The passage of an oral DNA vaccine through the gastrointestinal tract may be crucial because it is believed that intact antigen must reach the distal part of the gut to ensure uptake and transport by the epithelial cells. The strong responsiveness of the gut needs to be paid more attention as there is evidence that the gastrointestinal tract is an entry route for IPNV in Atlantic salmon [54], and that the virus can modulate the barrier function and transport activities of the intestinal epithelium.

The need for new insights into fish vaccination is evidence of the advance in our understanding of innate and adaptive immune responses. A recent review on IPNV vaccines [56] clearly shows that antigen delivery systems and routes of vaccine delivery influence the type of adaptive immune response generated in the host. Oral vaccines against IPNV and other viruses should be explored not only for their systemic immunity but also for the mucosal immunity they can induce. Determining the relevance of the gut as an immune-competent organ will be a promising area of study. Finally, our results indicate that as well as injection, incorporating the vaccine into food pellets seems to be a promising oral delivery method for the vaccination of cultured fish.

Acknowledgements

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2.2. Quitosano como alternativa para el recubrimiento de la vacuna DNA pcDNA-VP2 y su administración por vía oral.

El alginato de sodio es uno de los compuestos más utilizados en el recubrimiento de diferentes tipos de agentes activos, debido en parte a sus propiedades muco-adhesivas, con una alta estabilidad proteolítica, biodegradable, de baja toxicidad y costo relativamente bajo (Bowersock et al. 1999; Martins et al. 2007). El quitosano es un polisacárido catiónico (polisacárido) de glucosamina que presenta propiedades de muco-adhesión y es biodegradable. La fuente natural de este compuesto es el exoesqueleto en los crustáceos. En mamíferos, las partículas de quitosano han sido utilizadas para administrar fármacos por vía oral debido a su eficiencia en el transporte transcelular y paracelular de macromoleculas a través de la monocapa epitelial intestinal (enterocitos), placas de peyer y nódulos linfáticos mesentéricos (Desai et al. 1996). El rendimiento, porcentaje de carga y eficiencia en la encapsulación de las microesferas se ha descrito como 93,6%, 0,3% y 94,5%, respectivamente (J. Tian, Yu, et al. 2008).

El quitosano tiene propiedades que permite su uso en microencapsualación, es hidrófilo y ácido-soluble, por lo cual en entornos biológicos puede perder gran parte de su capacidad muco-adherente y volverse más permeable, liberando rápidamente los antígenos encapsulados (George and Abraham 2006). Por ello se requiere un refuerzo en el recubrimiento de las partículas, que le otorguen mayor estabilidad a ambientes ácidos; esto puede lograrse al suministrar alginato a la partícula, ya que este es un polímero que proporciona la estabilidad requerida. Además el alginato presenta interacciones electrostáticas favorables con las microesferas catiónicas de quitosano (Kang et al. 1996). La combinación de alginato y quitosano se seleccionó como constituyente estructural de otro tipo de microesferas ensayar. Se sabe que ambos compuestos pueden inducir una respuesta inmune separadamente, ya que han sido descritos como adyuvantes (Behera and Swain 2013). Las partículas resultantes (alginato-quitosano) en teoría podrían liberar mejor el pDNA, y mantener la integridad estructural del pDNA en ambientes ácidos durante más tiempo como ha sido descrito (Behera and Swain 2013). Dado que el tracto digestivo de los peces es un medio ácido y nuestra vacuna es oral, se realizaron ensayos en esta línea.

Para el recubrimiento del quitosano-alginato se utilizaron dos métodos diferentes de preparación de las microesferas como se describe en 2.2.1 métodos de encapsulación de vacunas DNA. Para este experimento se siguió el mismo esquema de incorporación de la vacuna en el pienso, y las truchas se alimentaron durante tres días sucesivos con este pienso “medicado”

Métodos de encapsulación de pcDNA-VP2 con alginato-quitosano.

La preparación de las microesferas de quitosano alginato fue realizada por los Dr. Carlos Elvira y Alberto Gallardo del Grupo de Funcionalización de Polímeros del

Instituto de Ciencia y Tecnología de Polímeros (ICTP-CSIC) Madrid. En esquema se realizó el siguiente procedimiento:

Preparación de microesferas de alginato-quitosano

Partiendo de las microencapsulaciones de las vacunas de DNA con alginato de sodio ya utilizado habitualmente (Ballesteros, Rodriguez Saint-Jean, et al. 2012; de las Heras et al. 2010), se llevó a cabo una gelificación externa con una disolución de $CaCl_2$, para reforzar las microesferas formando complejos polielectrolitos utilizando un polímero catiónico (quitosano). Las microesferas de alginato de sodio-quitosano fueron preparadas utilizando dos métodos, Quitosano 6 en un paso y Quitosano 7 en 2 pasos:

1) **Quitosano 6:** Se añadió 1.5 ml de DNA (pcDNA-VP2, pcDNA-) (1mg/mg) a 2.5 ml de alginato de sodio al 3%, agitando a 500 rpm durante 15 min. Por otra parte, se añadieron 0,5 ml de Span 80 a 100 ml de aceite de parafina y se agitó a 900 rpm. Se vertió gota a gota la mezcla de alginato+DNA sobre la mezcla de parafina+Span 80, manteniendo en todo momento agitación 900 rpm durante 30 min. A continuación se añadió 2,5 ml de una disolución de quitosano al 0,15% en ácido acético en el que se había disuelto $CaCl_2$ a 0,15M durante 2 h en agitación. Finalmente, la muestra fue centrifugada a 6000 rpm por 10 min y el sedimento se lavó con etanol al 70% hasta eliminar el aceite-. Las microesferas se liofilizaron durante 72 h. Posteriormente se resuspendieron en solución salina, se cuantificaron y almacenaron a -20ºC hasta su utilización.

2) **Quitosano 7**: Los primeros pasos descritos anteriormente son iguales, la variación con este método consiste en que una vez obtenida la mezcla del alginato, DNA, Parafina y Span 80 se le añadió 2,5 ml de $CaCl_2$ a 0,15 M y se agitó durante 2 h; al cabo de este tiempo se añadió 2.5 ml de quitosano 0,15% en ácido acético y se agitó durante 1h. Finalmente, la muestra fue centrifugada a 6000 rpm por 10 min y el sedimento se lavó con etanol al 70%, hasta eliminar el aceite. Las microesferas se liofilizaron, resuspendieron en solución salina, se cuantificaron y almacenaron a -20ºC hasta su utilización.

Determinación de tamaño de microesferas de quitosano por Microscopia electrónica.

Parte de las microesferas de alginato-quitosano liofilizadas fueron resuspendidas en agua y sonicadas en un sonicador de baño durante 30s. Luego, se depositó una gota de la suspensión sobre un porta de vidrio y se dejó secar, una vez secas las muestras se metalizaron con Oro “Au”. En las muestras se observan restos de aceite y se determinó el tamaño de las partículas, que varió entre 5 a 10 µm. (Ver figuras 2 al 5).

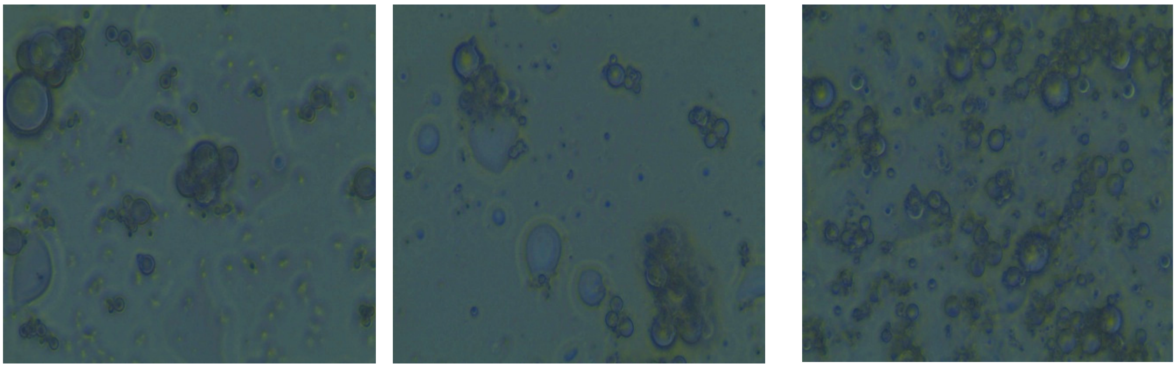


Figura 2. Microscopía óptica de las emulsiones. A) Alginato, B) Alginato-Ca, C) Alginato-Ca+quitosano. Aumento 40X. Método quitosano 6.

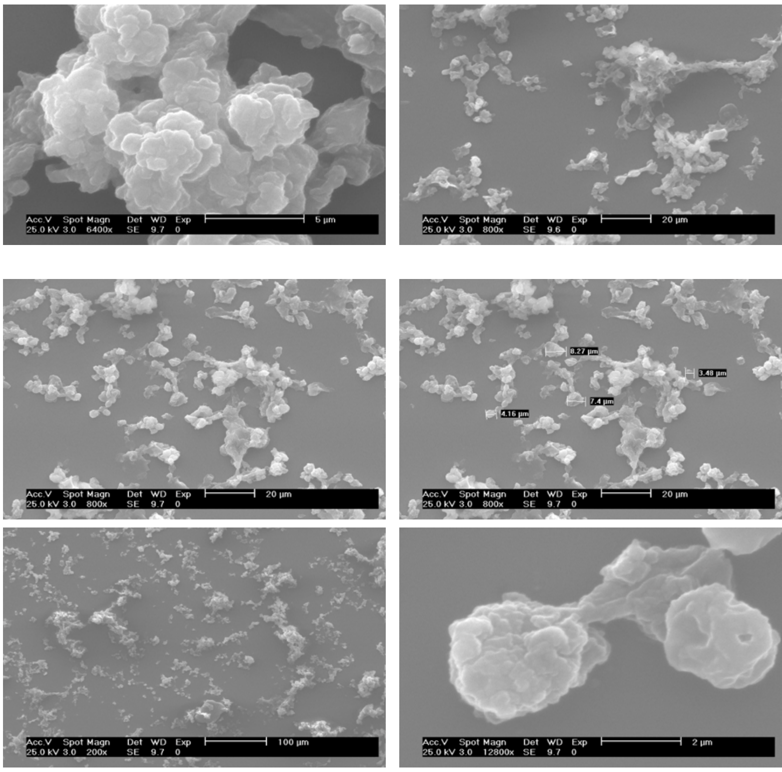


Figura 3. Microscopía electrónica de partículas de Alginato-quitosano-6 sin DNA.

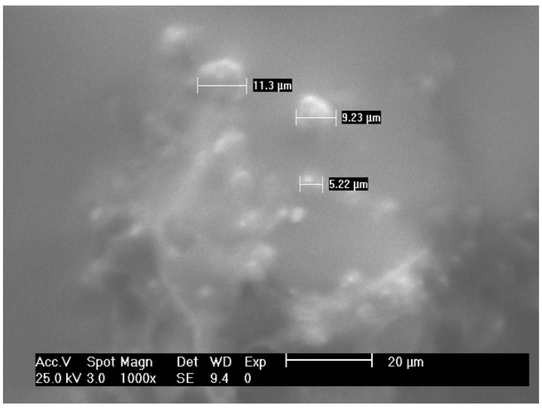


Figura 4. Microscopía electrónica de las partículas alginato-quitosano-6 con plásmido.

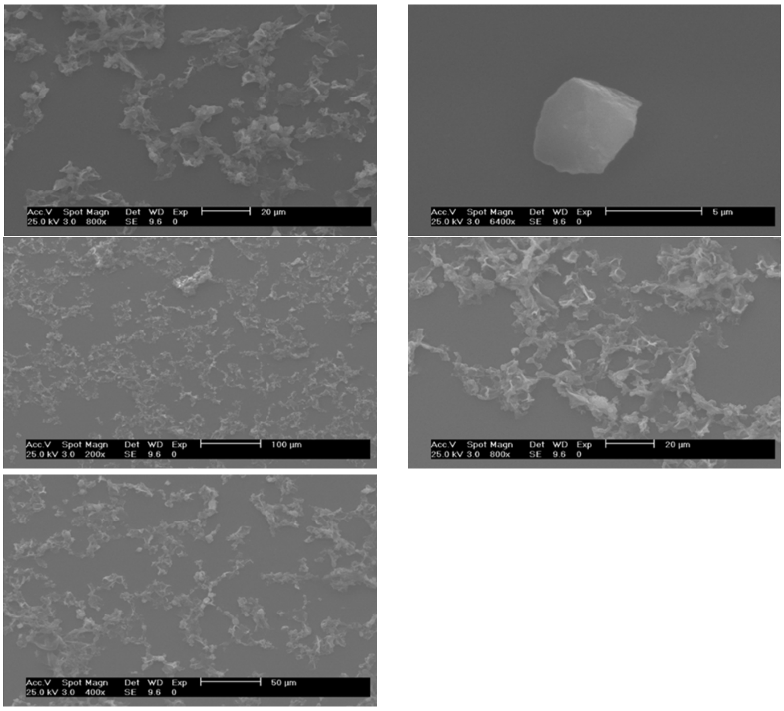


Figura 5. Microscopía electrónica de las partículas alginato-quitosano 7 con plásmido.

Diseño experimental:

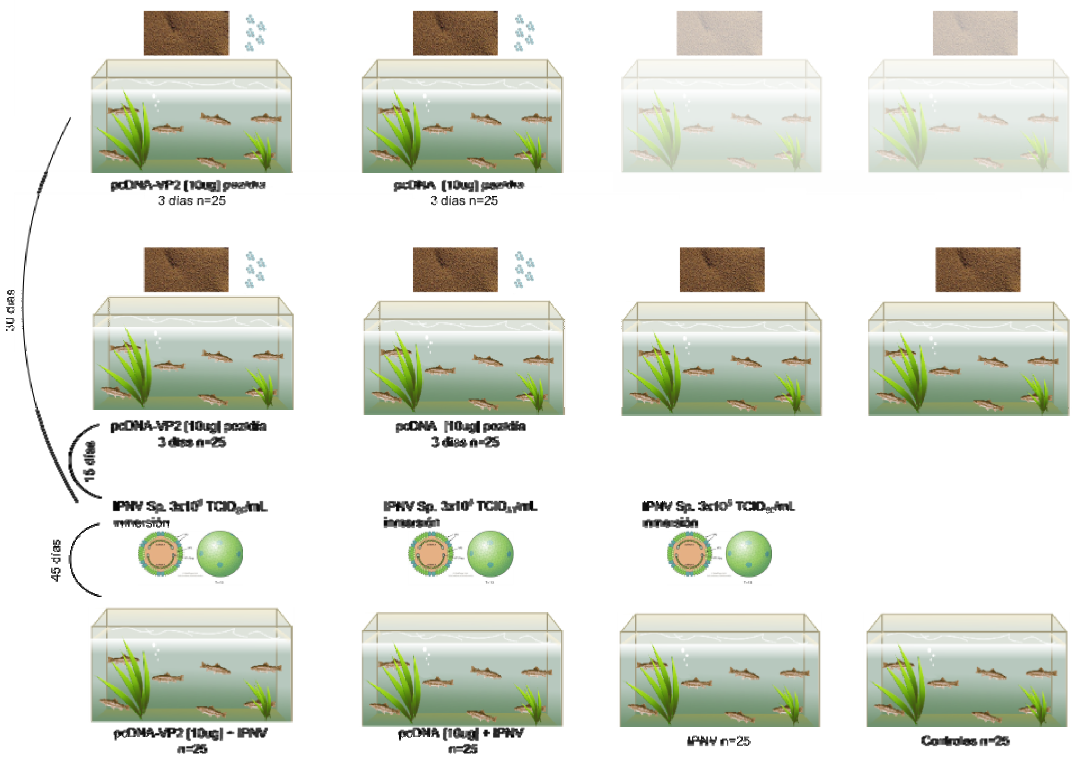


Figura 6: Esquema del diseño experimental.

Truchas arco iris de aproximadamente 1 g, 3.5 cm de longitud y 2 meses de edad

- Grupo de peces (n=20) alimentados con pienso comercial suplementado con 10 µg/día de pcDNA-VP2 recubierta con alginato y quitosano-6 durante 3 días "quitosano 6" A los 15 y 30 días post vacunación (dosis estimada por individuo), se infectaron los peces con 3 X 10^5 $TCID_{50}$/ml de IPNV Sp durante 2 horas por inmersión.
- Grupo de peces (n=20) alimentados con pienso comercial suplementado con 10 µg/día de pcDNA-VP2 recubierta con alginato y quitosano-7 durante 3 días "quitosano 7" A los 15 y 30 días post vacunación (dosis estimada por individuo), se infectaron los peces con 3 X 10^5 $TCID_{50}$/ml de IPNV Sp durante 2 horas por inmersión
- Grupo de peces (n=20) alimentados con pienso comercial suplementado con 10 µg de pcDNA-VP2 recubierta con alginato durante 3 días. "alginato" A los 15 y 30 días post vacunación (dosis estimada por individuo), se infectaron los peces con 3 X 10^5 $TCID_{50}$/ml de IPNV Sp durante 2 horas por inmersión.
- Grupo de peces (n=20) se infectaron con 3 X 10^5 TCI_{D50}/ml de IPNV Sp durante 2 horas por inmersión. "Infectados"
- Grupo de peces (n=20) sin tratamiento n=20 "Control".

Tiempos de muestreos: 3, 30 días post-vacunación y 10 días pos infección.

Muestras: Bazo, Intestino posterior, riñón anterior, hígado y branquias.

Métodos utilizados: Extracción de RNA total utilizando TriZol, síntesis de cDNA, PCR cuantitativa a tiempo real.

Resultados y discusión:

2.2.1. Cuantificación del gen VP2 en varios órganos de peces vacunados:

Se cuantificó la expresión del gen VP2 de IPNV por RT-qPCR en varios órganos de alevines de trucha vacunadas con 10µg de pcDNA-VP2 recubierta con alginato-chitosano (6 y 7) y alginato por vía oral con objeto de evaluar la eficiencia de los diferentes métodos de microencapsulación así como los niveles de absorción y distribución de la vacuna en los diferentes órganos. En la figura 7, se observa que a los 7 días, el transgen VP2 de la vacuna, presentó mayores niveles de expresión en todos los órganos evaluados cuando fue administrada mediante el recubrimiento de quitosano-7, comparado con los otros métodos de recubrimiento de la vacuna utilizados.

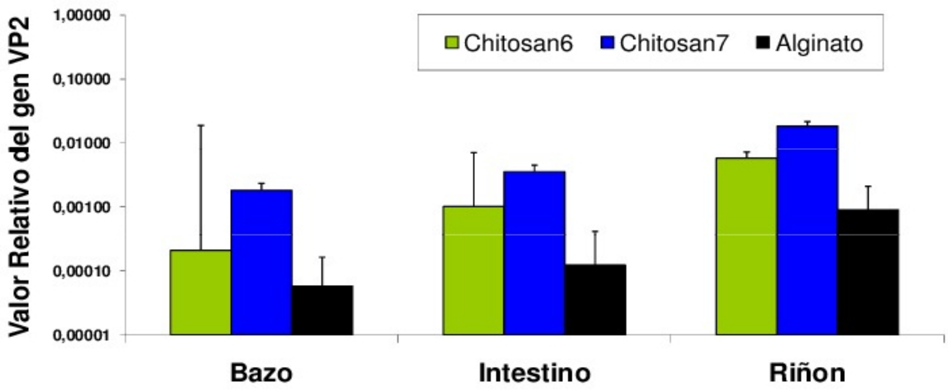


Figura 7: Cuantificación mediante qPCR del gen VP2 en diferentes órganos de peces vacunados por vía oral con la vacuna pcDNA-VP2 recubierta con quitosano-6 o quitosano-7 y alginato, a los 7 días. n=3.

2.2.2. Cuantificación del gen NS en varios órganos de peces vacunados e infectados:

La replicación viral en peces vacunados e infectados se estudió mediante la evaluación de la expresión del gen NS del IPNV en los principales órganos diana del virus, determinada por RT-qPCR. Los peces vacunados con pcDNA-VP2 con diferentes recubrimientos y peces sin tratamiento fueron infectados con IPNV. Tras 10 días de infección, se sacrificaron tres alevines por grupo, se extrajo RNA de diferentes órganos (riñón anterior, branquias, hígado, ciego pilórico y bazo) y por RT-qPCR se determinó la expresión del gen NS (VP4) del virus. En la figura 8 se observa que en todos los órganos estudiados la vacuna recubierta con alginato es la que presenta menores niveles del gen, lo que indica que, al menos en el momento de infección del IPNV seleccionado (10 días pv) es el alginato el sistema más eficiente para obtener disminuir la replicación viral, mientras que la vacuna administrada con el recubrimiento de “alginato quitosano”, presentan niveles elevados de este gen NS en todos los órganos evaluados.

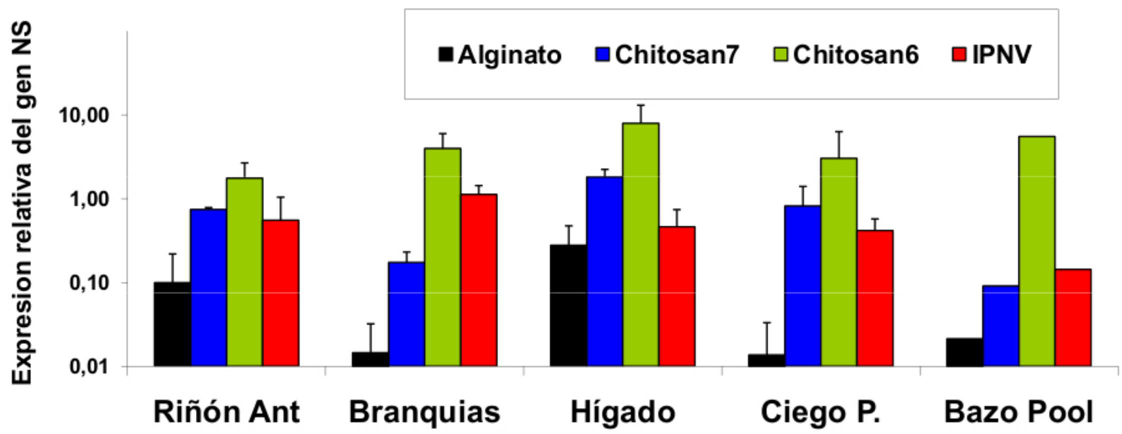


Figura 8: Expresión del gen NS en diferentes órganos de peces vacunados por vía oral con la vacuna pcDNA-VP2 recubierta con alginato-quitosano-6 o alginato-quitosano-7 y alginato e infectado con IPNV a los 10 días post-infección, n=3.

2.2.3. Expresión de genes inmunes en diferentes órganos:

Con objeto de conocer y comparar la eficiencia en la estimulación de genes inmunes en diferentes órganos al utilizar estos recubrimiento para la encapsulación de la vacuna DNA, se estudió la expresión de genes inmunes como IFNs, Mx e ILs en diferentes órganos, a los 3 días post vacunación. En intestino se produjo una respuesta inmune de mucosa al ser estimulado directamente por el plásmido pcDNA-VP2, y por la estimulación del material de recubrimiento de la vacuna, alginato y quitosano. En la figura 9 se observa que el intestino posterior es el segmento con mayor expresión de genes inmunes innatos como IFN-γ y Mx1. El IFN y otros genes que actúan en su ruta pueden codificar proteínas antivirales como Mx (en salmón, inhibe directamente la síntesis de proteínas de IPNV), todos los genes evaluados mostraron un incremento en la expresión tanto en órganos de mucosa y afectados directamente por la vía de la vacuna como en los órganos internos (bazo y riñón), siendo el intestino posterior el órgano con la más alta expresión de IFNγ y Mx. Respecto al recubrimiento de la vacuna DNA, se encontraron niveles de expresión génica similares en todos los órganos evaluados a los de 3 días transcurridos tras el último consumo de pienso con vacuna, especialmente para el IFNγ; sin embargo, en el caso de Mx1, la mayor expresión fue producida por quitosano-6 en el bazo e intestino en comparación de los peces vacunados con el plásmido recombinante recubierto con alginato. Por otra parte, para el gen mx1 se encontraron niveles similares en su expresión al recubrir la vacuna DNA con quitosano-7 o alginato.

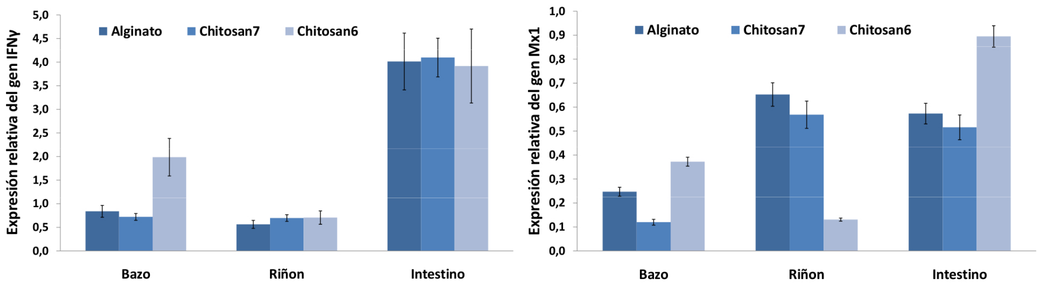
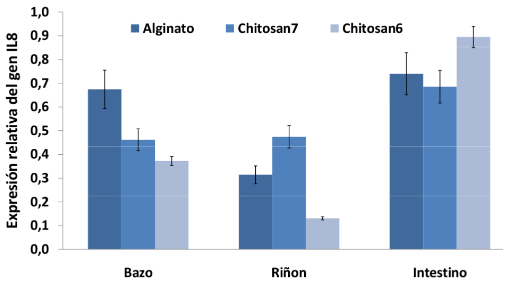
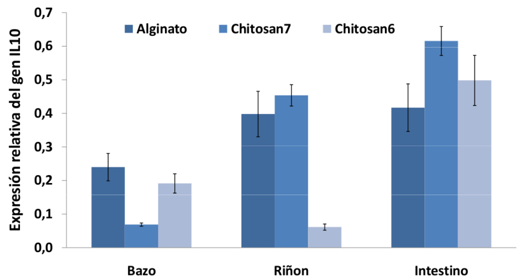


Figura 9: Respuesta inmune innata en bazo, riñón anterior e intestino de peces alimentados con pienso que contiene la vacuna pcDNA-Vp2 encapsulada en microesferas de alginato, quitosano-6 o quitosano-7 a los 3 días post-vacunación, n=3 peces.

El órgano con una mayor expresión de genes relacionados con interleuquinas es el intestino posterior. La expresión de la interleuquina IL8 fue alta en los peces vacunados con pcDNA-VP2+quitosano-6, respecto a los otros recubrimientos utilizados, y respecto a los otros órganos evaluados. El bazo también presento niveles altos de expresión de esta interleuquina, sin embargo, el mejor resultado fue obtenido al utilizar solo alginato. La interleuquina IL8 es quimiotáctica, por lo que atrae neutrófilos y ha sido descrita en truchas vacunadas frente a rhabdovirus por vía intramuscular; nuestros resultados indican que los niveles observados en el bazo pueden ser indicativos de la eficaz absorción de la vacuna oral en el intestino posterior.

En cuanto a la interleuquina IL10 anti-inflamatoria, presentó en todos los órganos niveles similares con los distintos recubrimientos utilizados. Finalmente en el intestino se produjo la mayor expresión de IL12b, seguido del riñón anterior; esta citoquina puede estar involucrada en la eficiencia de la protección que induce la vacuna, como se puede observar en la figura 10. La interleuquina 12B es una citoquina Th1 con cadena de heterodimero, conocida en mamíferos como factor estimulante de células asesinas naturales 2; es producida por los macrófagos, monocitos, células dendríticas y linfocitos B en respuesta a los patógenos intracelulares (Santos et al. 2007). Y ha sido utilizada como adyuvante de vacunas para incrementar la inmunidad en mucosas (Klas et al. 2002; Takakura et al. 2004).





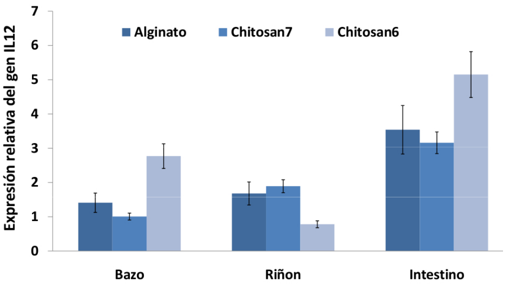


Figura 10: Respuesta de interleuquinas en órganos de peces alimentados con pienso que contiene la vacuna pcDNA-Vp2 recubierta en microesferas de alginato, quitosano-6 o quitosano-7, a los 3 días pv, n=3 peces.

Comprobada la capacidad de la vacuna alginato-quitosano para inducir respuestas inmunes, el siguiente paso consistió en evaluar en trucha arco-iris la protección de esta vacuna con diferentes coberturas frente a la infección.

2.2.4. Protección inducida por la vacuna pcDNA-VP2 encapsulada con diferentes recubrimientos, tras la infección por IPNV a los 15 días post-vacunación.

La eficacia de la vacuna pcDNA-VP2 encapsulada con diferentes recubrimientos frente al virus IPN se determinó según la mortalidad acumulativa de alevines vacunados e infectados comparándolos con los peces infectados sin vacunar (control virus). Con esos datos se determinó el índice de supervivencia relativa (RPS).

2.2.5. Mortalidad Acumulativa:

Los alevines vacunados por vía oral con alginato-quitosano 6, alginato-quitosano 7 y alginato (15 días pv.) y los sin vacunar (control virus) se infectaron con IPNV por inmersión. Los peces se observaron diariamente durante 30 días pi y se determinó la mortalidad acumulativa. La mortalidad acumulativa final en el grupo control fue del 75% y en los peces vacunados con pcDNA-VP2 recubierto con alginato-chitosano-6 fue del 30% (RPS=60%), con alginato-chitosano-7 fue del 20% (RPS=73.4 %) y en el grupo con alginato hubo mortalidad del 5 % (RPS=95%). Ver figura 11.

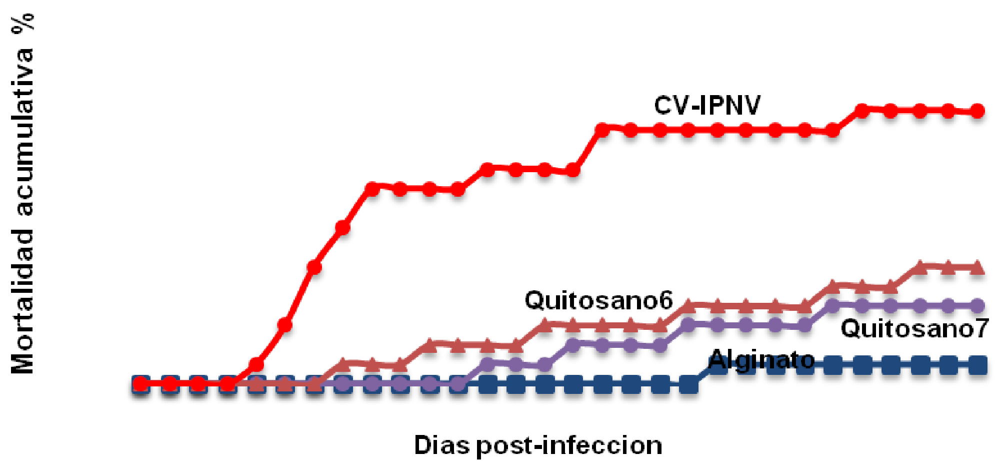


Figura 11: Mortalidad acumulativa de truchas vacunadas oralmente e infectadas con IPNV Sp., a los 15 días pv, y observadas durante 30 días. La vacuna se encapsuló con recubrimientos de alginato-quitosan-6, alginato-quitosan-7 y alginato solo, y se mezcló con pienso comercial.

Al no observarse una mejora significativa en el rendimiento en la protección de la vacuna administrada oralmente con diferentes recubrimientos, los experimentos siguientes con IHNV se realizaron con la fórmula ya probada de recubrimiento simple con alginato.

3. Determinar la eficacia de la vacuna pcDNA-VP2 oral en la prevención de estados de persistencia del virus de la Necrosis Pancreática Infecciosa.

3.1. Respuesta inmune de una vacuna oral “pcDNA-Vp2” en relación al estado de persistencia de IPNV en trucha arco iris.

Además de las altas mortalidades que el IPNV puede provocar en los peces infectados, los supervivientes se convierten en portadores asintomáticos del virus, que transmiten la enfermedad a otros peces expuestos y a su progenie. Los huevos embrionados y los productos sexuales contienen virus y son una fuente de dispersión de la infección. En este trabajo de tesis, se evaluó la eficacia de la vacuna pcDNA-VP2 para prevenir o disminuir la aparición de portadores del virus en peces en supervivientes a infecciones experimentales. Para ello, se vacunaron alevines de trucha con pcDNA-VP2; a los 15 días pv. se infectaron con virus IPN y, a los 45 días pi. se evaluó el estado de portador de virus de los supervivientes. Para lo cual, se determinó la presencia del virus y los niveles de expresión de transcritos virales en peces supervivientes vacunados e infectados, comparándolos en los infectados sin vacunar. Además en este trabajo se evaluaron los niveles de expresión de citoquinas y su correlación con la capacidad de los peces vacunados de establecer una respuesta inmune humoral y celular contra el virus, con objeto de conocer si los mecanismos de inmunoregulación pueden influir en el fenómeno de persistencia viral. En los peces supervivientes a la infección con IPNV, vacunados y control sin vacunar, se examinó la expresión de IFN-I, IFN-γ, TNF-α, IL8, IL10, IL12, MHC-I, IgM e IgT durante una infección en estado agudo (7 días) y en estado persistente (45 días).

Diseño experimental:

Alevines de trucha arco iris de aproximadamente 1.5 g, 3-4 cm de longitud y de 2 y 4 meses de edad

- Grupo de peces n=30 vacunados con 10μg de pcDNA-VP2 vía oral. A los 15 días pv. se infectaron por inmersión con 3×10^5 $TCID_{50}$/ml de IPNV Sp. “vacunados”.
- Grupo de peces (n=30) vacunados con pcDNA [10 μg] por vía oral. A los 15 dv. se infectaron por inmersión con IPNV Sp. “Plásmido vacío”.
- Grupo de peces (n=30) sin tratamiento “Control”.
- Grupo de peces (n=20) infectados por inmersión con 3×10^5 $TCID_{50}mL^{-1}$ de IPNV “Infectados”

Tiempos de muestreos: 10 y 45 días post-infección con IPNV.

Muestras: Riñón anterior, Bazo, Branquias, Ciego pilórico e Hígado.

Métodos: Extracción de RNA total utilizando TriZol, síntesis de cDNA, PCR cuantitativa a tiempo real.

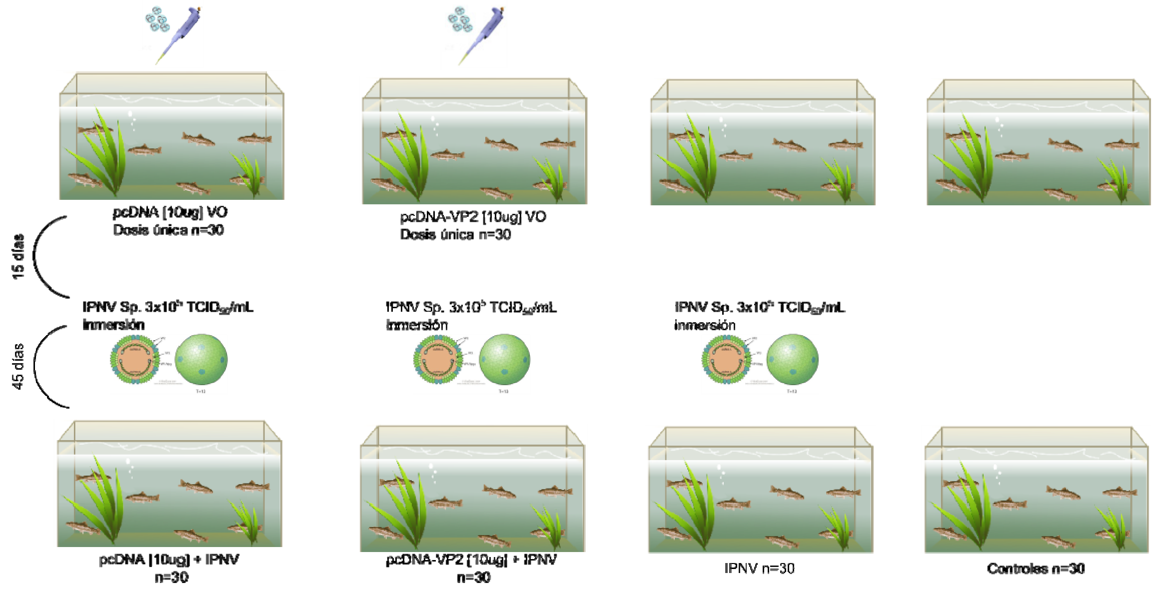


Figura 1: Diseño experimental

Resumen:

En este trabajo se determinó la capacidad antiviral de la vacuna durante 45 días post infección. Además, a los supervivientes vacunados y sin vacunar se les realizaron pruebas virológicas para detectar la formación de portadores frente al virus IPN. En las muestras de homogeneizados de órganos (riñón, hígado, bazo, intestino y branquias, procesados individualmente) de los supervivientes vacunados e infectados inoculados, que fueron inoculados en células susceptibles, no aparecieron efectos citopáticos de virus tras dos pases sucesivos. Sin embargo, en los cultivos inoculados con homogeneizado de órganos de peces infectados sin vacunar se aisló virus IPN.

Paralelamente se evaluó la expresión de antígenos virales en ambos grupos mediante la cuantificación de la proteína VP3 del virus por inmunofluorescencia indirecta, cuantificada por citometría de flujo. Además se comprobó que los niveles de expresión del gen VP4 del virus en los diferentes órganos (determinado por RTqPCR), a los 10 días pi, es significativamente menor en peces vacunados e infectados que en los controles infectados sin vacunar, indicando una disminución de virus infectivo achacable a la vacunación. Según los resultados obtenidos podemos concluir que la vacuna puede evitar la formación de portadores o disminuir su número. Un posible mecanismo sería la inducción de varios genes inmunomoduladores que conducen a una inhibición de la replicación viral, como se constata en los resultados de RTqPCR; de hecho los niveles de IFN-I son regulados incluso hasta 45 dpi en los peces vacunados e infectados en comparación con los peces únicamente infectados.

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Research paper

Immune responses to oral pcDNA-VP2 vaccine in relation to infectious pancreatic necrosis virus carrier state in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

The VP2 gene of infectious pancreatic necrosis virus, encoded in an expression plasmid and encapsulated in alginate microspheres, was used for oral DNA vaccination of fish to better understand the carrier state and the action of the vaccine. The efficacy of the vaccine was evaluated by measuring the prevention of virus persistence in the vaccinated fish that survived after waterborne virus challenge. A real-time RT-qPCR analysis revealed lower levels of IPNV-VP4 transcripts in rainbow trout survivors among vaccinated and challenged fish compared with the control virus group at 45 days post-infection. The infective virus was recovered from asymptomatic virus control fish, but not from the vaccinated survivor fish, suggesting an active role of the vaccine in the control of IPNV infection. Moreover, the levels of IPNV and immune-related gene expression were quantified in fish showing clinical infection as well as in asymptomatic rainbow trout survivors. The vaccine mimicked the action of the virus, although stronger expression of immune-related genes, except for IFN-1 and IL12, was detected in survivors from the virus control (carrier) group than in those from the vaccinated group. The transcriptional levels of the examined genes also showed significant differences in the virus control fish at 10 and 45 days post-challenge.

1. Introduction

Infectious pancreatic necrosis virus (IPNV) is the aetiological agent of infectious pancreatic necrosis disease, which causes high mortality in cultured salmonid fish. Although IPNV is not one of the disease agents listed by the World Organisation for Animal Health (OIE), it represents a problem for the freshwater aquaculture industry due to its worldwide dispersal and the important economic losses that it causes.

IPNV belongs to the *Birnaviridae* family and is the archetypical species of the *Aquabirnavirus* genus, whose members are naked, icosahedral viruses with a shell that is 60 nm in diameter and harbour a two-segmented, double-stranded RNA genome (Dobos, 1995). Genome segment A is approximately 3100 nucleotides (nt) in length and contains two partially overlapping open reading frames (ORF). One ORF is a short fragment encoding a 12–17-kDa protein designated VP5. The other, longer, ORF encodes a 106-kDa precursor protein that is co-translationally cleaved by the viral VP4 protease, generating the structural VP2 and VP3 proteins. The smaller genomic segment B is 2784 nt long

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and encodes VP1, the virion-associated RNA-dependent RNA polymerase.

IPNV infects young fish in fresh water and post-smolts shortly after transfer to seawater (Evensen, 2008; Ronneseth et al., 2013), resulting in an acute disease and high mortality. Following infection, the fish are life-long carriers of the virus, shedding it to their surroundings through their faeces, urine and reproductive tract, thereby transmitting the virus to their progeny and other susceptible fish. Therefore, broodstock carriage is considered a source of the virus for lethal infection of hatchery-reared fry (Wolf, 1988). Indeed, this virus has been successfully detected in macrophages from the head kidney (Johansen and Sommer, 1995) and in leucocytes in the blood and head kidney in survivors after infection (Rodriguez Saint-Jean et al., 1991). Commercial seawater salmon farms may also harbour IPNV carriers, and it appears that IPNV outbreaks in seawater may be more important economically than freshwater outbreaks in fry (Melby et al., 1991).

IPNV persistence was first described and studied in infected cell lines in the 1980s (Hedrick and Fryer, 1981, 1982; Hedrick et al., 1978; Okamoto et al., 1983). Interest in persistence has recently been renewed, given that more accurate analysis and quantification of target molecules is now possible using techniques such as real-time PCR (qRT-PCR), enabling connections with the immune response to be assessed (Kileng et al., 2007; Marjara et al., 2010; Rodríguez Saint-Jean et al., 2010). Indeed, this technique is particularly useful for the study of viral carriers. The presence of the virus within the immune cells themselves may significantly affect the functions of the cells involved in immunity. Therefore, the investigation of experimentally induced IPNV persistence in trout may shed light on viral relapse and immune modulation during long-term infection, in addition to allowing testing of antiviral therapies and new vaccines.

Oral DNA vaccines are now beginning to be tested in fish as a new strategy for improving viral fish vaccines (Hølvold et al., 2014). In this context, microencapsulation is a good choice for plasmid delivery. We have previously reported the activity induced by a DNA vaccine that expresses the VP2 gene of IPNV (de Las Heras et al., 2009). Protection as high as a relative per cent survival (RPS) of 83% was observed after oral vaccination of trout with the pcDNA-VP2 plasmid encapsulated with alginates (de las Heras et al., 2010), associated with the production of neutralising antibodies that lasted for at least 60 days. We subsequently studied the differential expression of immune-related gene transcripts on the 7th day following oral vaccination with this VP2 gene using a newly designed oligo-microarray of *Oncorhynchus mykiss* genes (Ballesteros et al., 2012a).

In this study, a group of previously identified up-regulated genes was selected for further study in the context of acute and chronic IPNV infections and in vaccinated fish. Moreover, we aimed to quantify the levels of IPNV in rainbow trout survivors among both virus control group and fish that were challenged with the virus after oral vaccination. IPNV was detected via qRT-PCR of the VP4 gene, a non-structural gene of IPNV, to determine whether the virus was present in the VP2-vaccinated fish. The immune responses induced in all the asymptomatic fish were also evaluated to better understand the carrier state and action of the vaccine. We analysed the possible resistance to the virus in the vaccinated fish by comparing the relative number of IPNV carrier fish as well as the expression of IFN genes and some interleukin (IL), immunoglobulin (Ig) and major histocompatibility complex (MHC-1) genes in the vaccinated and infected trout. The infective virus was recovered from infected fish survivors that did not exhibit clinical symptoms, but not from the vaccinated survivor fish.

2. Materials and methods

2.1. Cells and virus

The BF-2 cell line from bluegill fry (*Lepomis macrochirus* Rafinesque 1819, ATCC-CCL 91) was used in this study. The BF-2 cells were cultured as previously described (Rodríguez Saint-Jean et al., 2010). Briefly, the cells were grown at 25 °C in Leibovitz's medium (L-15, Gibco, Fisher Scientific, Madrid, Spain) supplemented with 100 IU mL^{-1} of penicillin G, 100 μg mL^{-1} of streptomycin, 2 mM L-glutamine and 10% foetal bovine serum (FBS: Gibco) or with 2% FBS in the maintenance medium (MM). The IPNV Sp strain (ATCC VR 1318) was used in this study. The virus was propagated in BF-2 cells at 19 ± 1 °C.

2.2. Fish

Rainbow trout (mean size and weight of 3.5–4 cm and 1.5 g, respectively) were purchased from a local spring water farm with no history of viral disease. The fish were kept under a 12/12 h light/dark regime at 15 °C in 350-L closed re-circulating water tanks (Living Stream, Frigid Units Inc., Ohio) at the Centro de Investigaciones Biológicas (CSIC, Madrid, Spain). The fish were fed daily with a diet of commercial pellets and were acclimatised to the aquarium system at least 2 weeks prior to experimentation. To assess their health, pools of five fish were examined for virus using standard protocols (Anonymous, 2012). Likewise, BF-2 cells were inoculated with dilutions of homogenates obtained from the fish, then incubated at 15 °C and observed daily for possible cytopathic effects (CPEs). None of the examined fish lots showed positive results. The described experiments complied with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals and were previously approved by the CSIC Ethics Committee.

2.3. Oral vaccine against IPNV

The DNA vaccine (pcDNA-VP2) was prepared as previously described (de Las Heras et al., 2009), with the IPNV-VP2 gene inserted into the pcDNA.3.1/V5/His-TOPO vaccine vector (Invitrogen, USA). The pcDNA-VP2 and pcDNA plasmids were coated with alginate; these microspheres were prepared as described elsewhere (de las Heras et al., 2010).

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2.4. Experimental design

Vaccinated and mock-vaccinated fish challenged with IPNV at 15 days pv were observed for 45 days post-challenge, and the following assessments were then performed: (1) cumulative mortality and the relative per cent survival; (2) viral persistence, by quantification (RT-qPCR) of VP4 viral gene expression in organ samples; (3) the levels of VP3 IPNV antigens in fish survivors, by flow cytometry; (4) isolation of the virus in cell cultures; and (5) the expression levels of IFN and other immune-related genes. The expression levels of these genes in fish with sub-clinical infections (45 days post-challenge) were compared with those in fish that were sampled at 10 days post-challenge when the infection was clinically evident. A sampling periods of 10 days was considered here because some genes related to both innate and specific immune responses have been previously detected at approximately 7–15 days post-challenge (de las Heras et al., 2010; Ballesteros et al., 2014).

2.5. Immunisation

The trout were distributed in separate 45-L tanks (25 fish each), supplied with non-chlorinated water using exterior carbon filters (Eheim, Germany) and additional aeration. The experiment included three treatment groups: fish that were orally vaccinated with 10 μg of DNA/plasmid per fish diluted in 10 μL of saline buffer (pcDNA-VP2 group); fish that received similar amounts of microspheres containing the empty plasmid (pcDNA group); and mock-vaccinated control fish. Vaccination was performed with an automatic 20-μL pipette tip as previously described (Ballesteros et al., 2012a; de las Heras et al., 2010). The water quality was maintained at optimum levels, and the conditions in all the tanks were equivalent.

2.6. Challenge with IPNV and sampling

At 15 days post-vaccination (pv), the fish (two tanks per group, 20 fish per tank) were challenged via immersion with IPNV at a dose of 5×10^5 $TCID_{50}$ mL^{-1} for 2 h at 15 °C in a total volume of 4 L of dechlorinated and aerated water per tank. The fish were held in 45-L aquaria, and mortality was recorded daily for 45 days post-infection (pi). The fish surviving at 45 days post-challenge when mortality ceased were sampled. At the time of sampling, the fish were sacrificed with an overdose of MS-222, and the liver, spleen, kidney, pyloric caeca and gill tissues were removed aseptically and preserved in RNAlater (Ambion Inc.). Samples of visceral pools were also collected for virus isolation in cell cultures.

Cumulative mortality was also assessed in parallel experiments. The mortality in the tanks was recorded daily, and the experiment was evaluated at 45 days post-challenge. The RPS was then calculated using the formula RPS = [1 − % (mortality of fish given vaccine/% mortality of fish given pcDNA)] × 100. The identity of IPNV in the dead fish was confirmed through seroneutralisation with polyclonal antiserum or through amplification of the VP4 viral gene.

2.7. RNA extraction, cDNA synthesis and real-time PCR (qPCR)

The organ tissue samples stored at −20 °C in RNA later were homogenised in a TissueLyser Lt (Qiagen, Madrid, Spain). RNA was extracted using TRIzol LS (Invitrogen) and treated with DNase I according to the manufacturer's instructions. The concentration and purity of the RNA were measured spectrophotometrically (NanoDrop Technologies, USA, -www.nanodrop.com-), and for cDNA synthesis, total RNA (5 μg) was primed with 25 pmol/μL of oligo-d(T) and reverse-transcribed using the Super Script™ II Kit (Invitrogen, USA). The obtained cDNA was stored at −20 °C for further experiments.

Real-time PCR amplification was performed in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Madrid, Spain) using the Quantimix Easy Kit (Biotools Labs, Spain) and SYBR green, as described elsewhere (Ballesteros et al., 2012b,c). Each amplification reaction (20 μL) contained 10 μL of 2× Quantimix Easy Master Mix, 2 μL of cDNA, 1 μL (10 mM) of forward and reverse primers and 6 μL of nuclease-free water. Negative controls without the template (cDNA) were included. Elongation factor 1α (EF-1α) was used as a housekeeping control gene, and all the PCR amplifications were performed in duplicate. The cycling conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. After a run, melting curve analyses were performed for each amplicon to ensure the specificity of the primers. The obtained data were analysed using iQ5 optical system software, version 2.0 (Bio-Rad), and the expression of the target genes was corrected based on the endogenous control expression (EF-1α) and calculated as the relative values ($2^{-\Delta CT}$) or fold change in relative expression according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.8. Persistence of IPNV: detection of VP4 mRNA expression

The VP4 gene was quantified via RT qPCR as previously described (Ballesteros et al., 2014). The fish surviving at 45 days pi were sacrificed, and their kidney, gill, liver, pyloric caeca and spleen tissues were removed aseptically. Then, total RNA was isolated using the TRIzol reagent (Invitrogen, USA), from which cDNAs were synthesised (primers in Table 1).

2.9. Detection of IPNV antigens in fish survivors by flow cytometry

Flow cytometry assays were performed in BF-2 cells inoculated with homogenates of organs from asymptomatic surviving trout at 45 days pi. The fish were examined through indirect immunofluorescence, and the percentages of cells expressing the IPNV-VP3 protein were determined as previously described (Pérez et al., 1994; Rodriguez et al., 2001). Briefly, the organs from three fish were individually homogenised in L-15 medium supplemented with 2% FBS, followed by centrifugation at 800 × *g* for 15 min. BF-2 cells were inoculated with different dilutions of the supernatants and incubated at 20 °C. After 3

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Table 1
List of primer pairs designed for gene expression analysis by qRT-PCR.

Genes	Primer sequence 5′–3′: Forward Reverse	Accession Number
IFN1	AAAACTGTTTGATGGGAATATGAAA CGTTTCAGTCTCCTCTCAGGTT	NM_001124531
IFNγ	CTGAAAGTCCACTATAAGATCTCCA CCCTGGACTGTGGTGTCAC	FM864345.1
IL8	GAATGTCAGCCAGCCTTGTC TCCAGACAAATCTCCTGACCG	AJ279069
IL10	CGACTTTAAATCTCCCATCGAC GCATTGGACGATCTCTTTCTT	AB118099
IL12b	ATGTGGTTACGGGAGGC ATGTGGTTACGGGAGGC	AJ548830.1
TNF2	TGCTGCTCCATGTGTGGTGC AGGGACGGGGAGCCTTGAT	DQ218473.1
MHC*1 uda-hc*	GCAACCCAATTTCATGCAGG ACACTCAATGCAGGTCTGGG	EU036638.1
IgM	ACCTTAACCAGCCGAAAGGG TGTCCCATTGCTCCAGTCC	X65263.1
IgT	AGCACCAGGGTGAAACCA GCGGTGGGTTCAGAGTCA	AY870265
EF1α(house-keeping gene)	GATCCAGAAGGAGGTCACCA TTACGTTCGACCTTCCATCC	AF498320
VP4	AGGAGATGACATGTGCTACACCG CCAGCGAATATTTTCTCCACCA	M18049.1

days, the BF-2 cell monolayers were enzymatically disaggregated, fixed with 3.7% formaldehyde in PBS for 15 min, washed twice in PBS and permeabilised with 0.01% Triton X-100 in PBS for 1 min. The cells were then incubated for 30 min at room temperature with a 1:10 dilution of an anti-IPNV-VP3 monoclonal antibody mAb 2F12 (Dominguez et al., 1990) (courtesy of Dr. Julio Coll, INIA, Madrid), after which they were washed in PBS that was supplemented with 2% FBS and incubated for 30 min at room temperature with a FITC-conjugated goat anti-mouse IgG antibody (Sigma, Spain). The fluorescence of the cell suspensions was measured on a Coulter XL flow cytometer (Beckmann-Coulter, Spain), determining the background fluorescence from uninfected cells and infected cells that were stained with an irrelevant antibody.

2.10. Virus isolation in fish survivors

The remaining virus was isolated from survivors using standard methods, and dilutions of the obtained samples (1:100) were then inoculated into BF-2 cell monolayers grown in 24-well plates (Falcon, Becton-Dickinson, France). The monolayers were incubated at 19 ± 1 °C and observed daily for CPEs. The samples were considered negative when no CPEs were observed after two passages.

2.11. Expression of immune-related genes in vaccinated or infected fish and in IPNV infection survivors

In an earlier study, we analysed gene expression in rainbow trout at seven days pv with the pcDNA.VP2 vaccine using a specific microarray (Ballesteros et al., 2012a). Some of the genes that were previously shown to be differentially expressed (>2-fold) following oral VP2-vaccination were evaluated in trout to determine the immunomodulatory ability of the virus or the vaccine during the early and late stages of infection. In this study, the fish were first processed when the virus begins to cause early deaths (10 days pi) and again at 45 days post-challenge when fish did not exhibit clinical symptoms. No virus was recovered from the vaccinated trout in cell cultures. The primers and probes that were used to analyse these genes via RT-qPCR are described in Table 1. The assessed genes were IFN; cytokine/interleukin-related genes, such as IL8 (Sanchez et al., 2007), IL10 (Inoue et al., 2005), IL11 (Wang et al., 2005), the p40 b chain of the IL12 heterodimer (Fischer et al., 2006) and tumour necrosis factor (TNF2), similar to TNFα (Zou et al., 2002); and genes related to adaptive immune responses, such as MHC1 (Hansen and LaPatra, 2002), IgM (Lorenzen et al., 1993) and IgT (Zhang et al., 2010).

2.12. Statistical analysis

Prior to the statistical analyses, the normality of the distribution of the data was checked and confirmed using the Shapiro–Wilk test. The data are presented as the mean ± standard deviation of the results from three trout. An analysis of variance (factorial ANOVAs) was run to determine whether the differentially expressed genes differed between the replicates for an individual gene, followed by Tukey's multiple comparison test for differences between the vaccinated group and the IPNV-infected group. Student's *t*-test was used to compare some of the paired samples. All the statistical analyses were run in SPSS version 15. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Fish mortality

Seven days post-challenge, the fish from the mock-vaccinated virus control group (IPNV) began to die as well

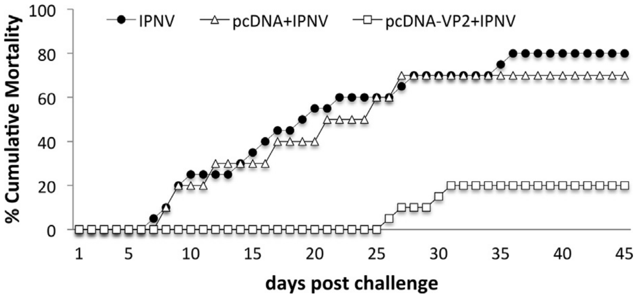

Fig. 1. Percentage of accumulated mortality of rainbow trout vaccinated and challenged with IPNV after 15 days (pcDNA-VP2 + IPNV). Non-vaccinated and challenged fish were maintained as virus control group (IPNV) as well as fish receiving the empty plasmid (pcDNA + IPNV). The mortality of 20 fish in each of two tanks per group is indicated.

as the fish that received the empty plasmid (pcDNA + IPNV). High cumulative per cent mortality was observed among these fish, along with additional signs of disease from the 7th to 20th day pi, consistent with viral infection. The surviving fish (around 20% and 25%, respectively) were apparently healthy at 45 days pi. In contrast, trout deaths were first observed in the vaccinated group on the 25th day post-challenge, and only 20% mortality was recorded at the end of the experiment (Fig. 1). The survivors of the infection from both the pcDNA-VP2-vaccinated and virus control groups exhibited no symptoms of disease. The fish were sampled at 45 days post-challenge when death had ceased and were processed for the presence of IPNV to investigate their potential as virus carriers.

3.2. Persistence of IPNV in the fish survivors

The expression of the VP4 gene was determined via RT-qPCR and used as a measure of IPNV replication or the viral load (Fig. 2). The relative expression of the VP4 gene was found to be increased significantly in the IPNV control group, whereas VP4 was only weakly expressed in the pcDNA-VP2-vaccinated fish. The surviving fish from the virus control group showed stronger expression in the gills and liver, indicating that these organs are targets of viral infection, at least during the chronic stage of infection. Weaker but detectable expression was recorded in the pyloric caeca and kidney, and the lowest expression was detected in the spleen. However, in the vaccinated fish, the protection induced by the pcDNA-VP2 vaccine was demonstrated by the very low or non-detectable expression of the target gene in the examined organs.

These results indicate that there were significantly lower levels of the virus in the vaccinated fish ($P < 0.05$).

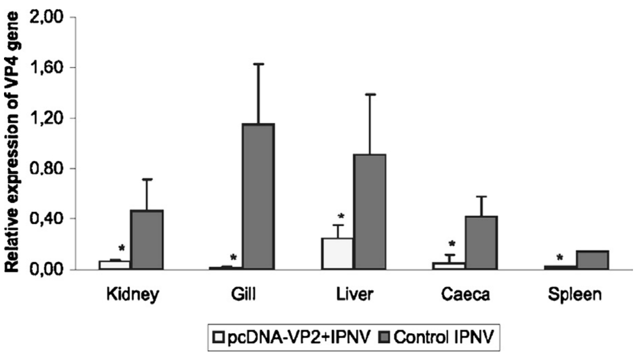

Fig. 2. Effect of the IPNV vaccine on the IPNV carrier state in vaccinated and unvaccinated fish. The IPNV load was determined through the expression of the non-structural VP4 gene analysed by real-time RT-PCR, evaluating its transcription in the kidneys of surviving fish at 45 days pi. The data is derived from three independent experiments ± standard deviation (SD) for three individuals from each group: *statistically significant differences at $P < 0.05$.

3.3. Expression of IPNV viral antigens in organs from surviving fish, quantified by flow cytometry

The recorded percentage of fluorescent cells represents the cells in which IPNV antigens (VP3) were expressed, as detected in the indirect immunofluorescence assay (Fig. 3). The BF-2 cells inoculated with samples of different organ homogenates from vaccinated and challenged fish showed either negative results or very low levels (ranging from 1.1 to 4%) of fluorescent cells (Fig. 3A). These results are consistent with the weak expression of the VP4 gene observed in the surviving fish and the non-recovery of the virus from the inoculated BF cell monolayers. In contrast, the BF-2 cells inoculated with gill and kidney homogenates from the virus control fish exhibited the greatest number of positive fluorescent cells, ranging from 65 to 86% (Fig. 3B) and showing mean values of approximately 73 and 62% fluorescent cells, respectively (Fig. 3C). The spleen, liver and kidney homogenate samples inoculated onto the BF-2 monolayers also resulted in a high percentage of fluorescent cells (from 43 to 49%). These results indicate that despite the absence of clinical symptoms in the IPNV control group of fish, the virus remained virulent, maintaining its capacity to infect and multiply in sensitive cells.

The histograms shown in Fig. 3C represent the average levels and standard deviations of the percentages of fluorescent cells that were detected in the examined samples and demonstrate the existence of significant differences between the unvaccinated and vaccinated fish.

3.4. Recovery of the virus from fish (vaccinated and control) at 45 days post-infection

Among the seven fish that survived in the virus control group, five produced a positive CPE in the cultured cells after 24–48 h pi at the first passage, along with most of the samples from fish from the pcDNA + IPNV group (Table 2). In contrast, none of the homogenates from the pcDNA-VP2 + IPNV (vaccinated fish) group produced a positive CPE at the first passage in BF-2 cells.

The samples that failed to produce a positive CPE were recovered from the cell cultures and re-inoculated into new cell monolayers to perform a second blind passage to amplify any potential small amounts of remaining virus. All the samples from the virus controls that failed to produce a positive CPE in the initial assay generated a CPE in the second passage, while the samples from the vaccinated group still failed to produce a CPE. The identity of IPNV was confirmed via seroneutralisation or RT-PCR assays and sequencing of the amplified product (data not shown).

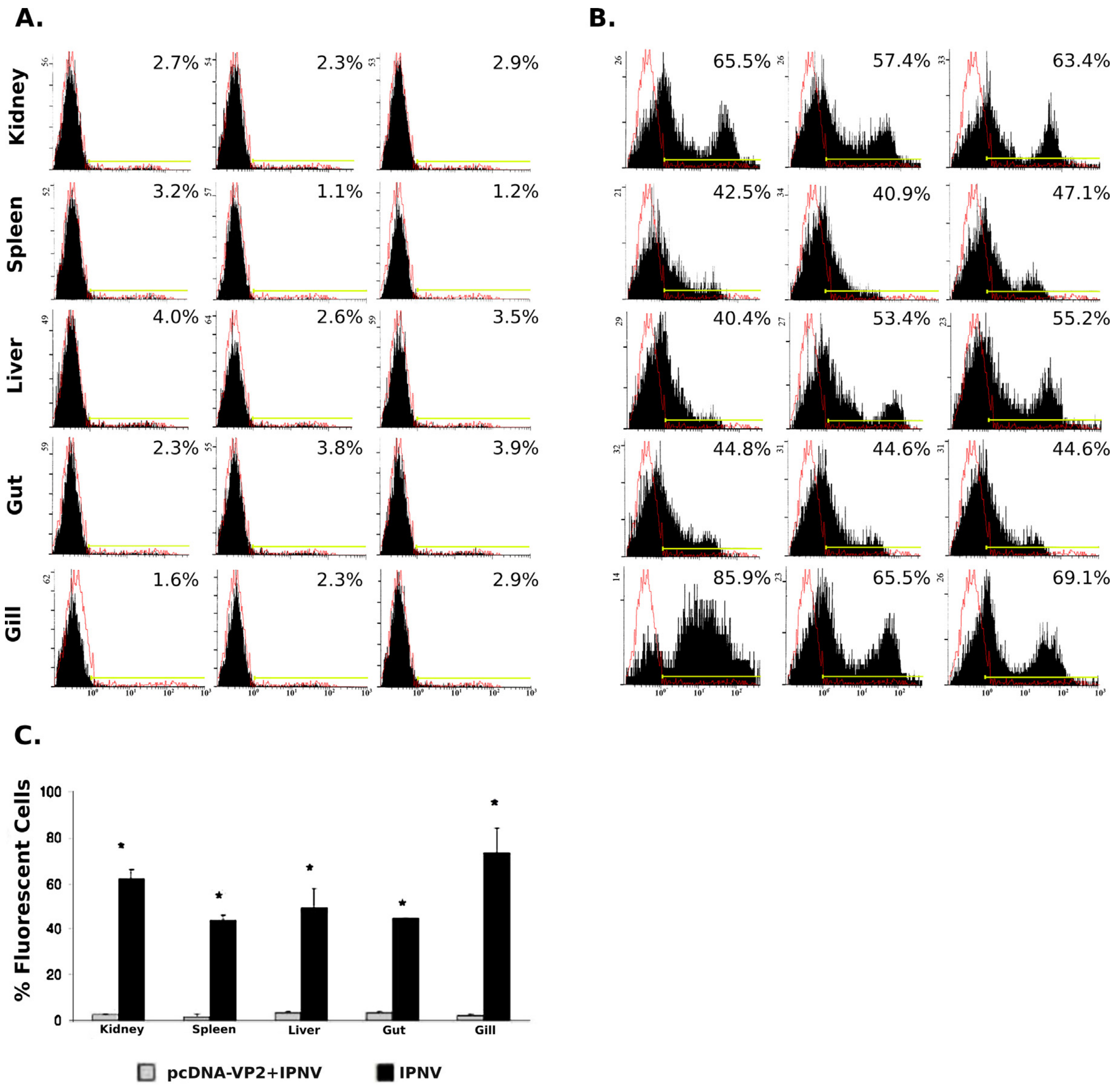

Fig. 3. Flow cytometry histograms (or percentages) of viral antigen expressing BF-2 cells infected with homogenised tissue from fish surviving IPNV infection for 45 days. (A) pcDNA-VP2-challenged group; (B) virus control group; background fluorescence is indicated. (C) Mean relative expression ± SD for three individuals from each group. *Statistically significant differences at $P < 0.05$.

3.5. Expression of immune-related genes in vaccinated or infected fish during the early and late stages of infection

The quantification data from the RT qPCR assays are shown in Fig. 4. The comparative results between the groups are expressed in fold values and summarised in Fig. 4 B1 (for the pcDNA-VP2-vaccinated fish relative to the virus control fish at 45 days pi) and Fig. 4 B2 (for the virus control fish at 45 days pi (i.e., "carriers") relative to the virus control fish at 10 days pi).

Greater up-regulation in the virus control fish was recorded for the IL8 gene at 10 days post-challenge, when the relative expression value for this gene had increased early to 32.44 ± 12.84, while in the vaccinated fish the relative expression value was 9.34 ± 4.92. Up-regulation of IFN-1 was observed at 45 days post-challenge in the vaccinated fish (1.67 ± 069), which was the only instance in which a gene showed higher expression compared with its level in the IPNV control fish (0.97 ± 0.2). These results agree with those previously obtained at 7 days (Ballesteros et al., 2012b,c). Comparative analysis of gene expression in the vaccinated fish and the virus control group at 45 days post-challenge revealed that only two genes, IFN-1 and IL12b, were up-regulated in the former group (Fig. 4B).

In the fish from the virus control group that were examined at 10 and 45 days post-challenge (Fig. 4A and B2), the transcription levels of the IFN-γ, IL-8, IL-12b, and IgM genes were higher at the earlier time point, when the virus was replicating, and the fish exhibited clinical symptoms (affected fish darkened, swam in spirals, and

Table 2
Isolation of IPNV in BF-2 cells from vaccinated and non-vaccinated rainbow trout that survived 45 days after challenge. Effects of the vaccine in viral persistence.

Fish	Virus Control[a] (CPE)[*]		Vaccinated with empty plasmid and infected[b] CPE		Vaccinated with pcDNA.VP2 and infected[c] CPE	
	P_1	P_2	P_1	P_2	P_1	P_2
1	+	+	–	+	–	–
2	–	+	–	+	–	–
3	+	+	–	+	–	–
4	–	+	+	+	–	–
5	+	+	+	+	–	–
6	+	+	+	+	–	–
7	+	+	+	+	–	–

[a] The fish were infected by immersion with IPNV, survivors sacrificed at 45 days pi and visceral homogenates were processed as indicated in Section 2.
[b] Fish vaccinated orally with pcDNA empty plasmid and infected after 15 days.
[c] Fish vaccinated orally with pcDNA.VP2 plasmid and infected after 15 days. Survivors at 45 days post infection were processed as in a.
[*] CPE, cytopathic effects on the monolayers; P1, P2, successive passages on cell monolayers.

developed exophthalmia and abdominal distension). In contrast, the induction of TNF-2α, IL10, MHC-I and IgT was higher at 45 days post-challenge, when the virus was most likely persistent. The increase in IL10 expression recorded in the IPNV survivors was the greatest among all the examined genes at 45 days post-challenge. Except for IL-12 and IgM, the differences in the expression levels of the examined genes at 10 and 45 days were significant.

The expression of genes related to mucosal immunity is of interest because the vaccine is administered orally. Therefore, the effect of the virus and the vaccine on IgM and IgT transcripts was evaluated in the surviving fish (Fig. 4).

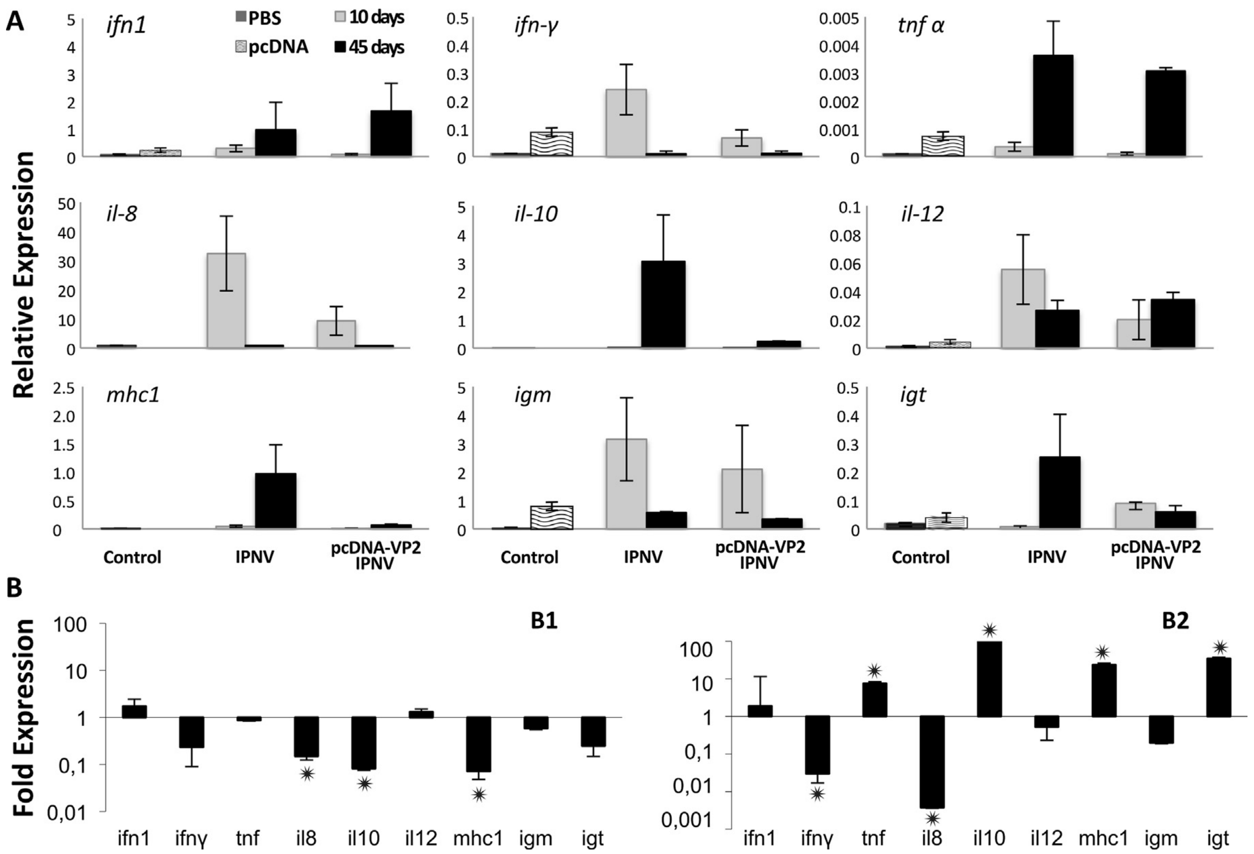

Fig. 4. Expression of immune-related genes in rainbow trout at 10 days pi and in survivors at 45 days pi. Samples were processed from vaccinated and challenged fish (pcDNA-VP2 + IPNV), virus control group (IPNV), mock-vaccinated fish (PBS) and fish vaccinated with the empty plasmid (pcDNA). The RT-qPCR data are presented as the normalised mean relative expression (±standard deviation) in three anterior kidneys from fish that were processed at 10 and 45 days pi (A). (B1) and (B2) summarise the comparative changes in gene expression observed in each group of fish. The histograms in (B1) represent the fold-change in expression in the pcDNA-VP2-vaccinated fish that survived to 45 days pi relative to that in the surviving IPNV control fish at this time. (B2) The fold change in gene expression in the IPNV control group at 45 days pi relative to that at 10 days pi. *Statistically significant differences at $P < 0.05$.

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Constitutive expression of these genes was detected in the head kidneys of the trout. In the virus control fish, the transcription level of IgM at 10 days post-challenge was significantly higher than most of the levels recorded for the other genes (except for IL8) and was higher than the expression level of the same gene at 45 days. In contrast, IgT expression was greater at 45 days post-challenge. The vaccinated fish showed similar patterns of expression to the virus control fish for the IgM and IgT genes, although at lower levels.

4. Discussion

This study showed that in the asymptomatic surviving trout that were vaccinated and then IPNV-challenged, the viral load and infectivity had been lost at 45 days post-challenge. The vaccine displayed strong antiviral activity, measured based on the reduction of virus VP4 transcript levels. The results agree with previous studies in which the vaccine was administered in food pellets (Ballesteros et al., 2014). The absence of positive isolation results after two successive passages in cell cultures is sufficient to consider a sample negative following standard diagnostic protocols. Thus, the vaccine induces several different immunomodulatory effects that result in inhibition of viral replication. The results of flow cytometry analysis also supported the existence of such inhibition, as the cells inoculated with homogenate samples from vaccinated fish did not show significant levels of viral antigens. In contrast, our observations indicated that the virus remained infective in the surviving IPNV control group from which it was recovered in cell cultures; there are two peaks in fluorescence intensity, one contains fewer cells but with a much stronger fluorescence intensity, probably those in which the virus is accumulating after replication.

Our RT-qPCR results demonstrated that IFN-1 transcript levels were up-regulated at 45 days post-challenge in the vaccinated fish compared with the non-vaccinated fish, with as were those of the IL-12 gene, which constituted an exception to the observed pattern, as most of the examined genes showed higher expression levels in the non-vaccinated infected fish (virus control) at 45 days post-challenge. The relative expression values for the selected genes were low in survivors, and most of the genes exhibited higher levels in the virus control trout than in the vaccinated trout. It appeared that the observed immune responses were similar to those found in mammals, where, once a vaccine has established certain mechanisms of protection, the responsiveness to the virus diminishes.

It is well established that the type I IFN system participates in the first line of defence against viral infections, and many reports have described its multiple functions in innate and adaptive immune responses (Das et al., 2007; Robertsen, 2006; Robertsen et al., 2003; Zou and Secombes, 2011). The roles of IFN type 1 in the virulence of IPNV or in the establishment of virus persistence have been discussed previously (Basanta et al., 2009; Collet et al., 2007; McBeath et al., 2007), although the mechanism underlying persistent IPNV infection is, to a large extent, unknown. The importance of IFN in establishing or maintaining viral persistence has been explored for other viruses relevant to fish aquaculture, such as betanodavirus nervous necrosis virus (Wu and Chi, 2006). In addition, the induction of a strong IFN response with poly I:C in carrier fish was not found to suppress the IPNV carrier state of Atlantic salmon broodstock (Lockhart et al., 2004).

The results of the present study agree with these earlier data to some degree, as IPNV could be detected in several organs of fish virus control that survived to 45 days pi. Neither the expression of IFN nor that of other related genes eliminated the virus, which could be re-isolated from the asymptomatic fish in this group. The question is different for the pcDNA-VP2-challenged fish, which showed slightly higher expression of IFN than the IPNV control fish at 45 days post-challenge. The virus not only disappeared from the surviving fish, in which very low expression of the VP4 gene was recorded, but its infectivity was also lost. pcDNA-VP2 is a DNA vaccine, and expression of the VP2 gene is expected to occur continuously for at least 60–90 days (de las Heras et al., 2010); thus, stimulation of IFN and other immune-related genes could be induced for longer periods than that for which the virus is present, possibly contributing to viral elimination or removal. In addition, persistence is not a well-understood process in viral infection, and many genes may be involved in the maintenance of a sub-clinical infection.

In contrast to IFN-1 (which is considered a marker of the innate immune response), the level of IFN-γ (which is involved in adaptive and specific immunity) was quite low in the IPNV carrier trout and was almost undetectable in the vaccinated and IPNV survivor fish at 45 days post-challenge. Marjara et al. (2011) reported similar results for the expression of IFNs in Atlantic salmon survivors of IPNV infection. While no significant differences were found in the expression level of IFN-γ relative to basal levels, a significant increase in the expression of IFN-1 was recorded.

There is little information regarding pro- and anti-inflammatory cytokines in fish and their relationship with viral infection and/or vaccination. In microarray assays we previously performed in vaccinated fish, several interleukin (IL) genes were found to be over-expressed, some of which were selected for comparison in the vaccinated and control fish (Ballesteros et al., 2012a,c,d) Here, we demonstrate that the expression level of the anti-inflammatory cytokine IL10 was different in the IPNV-infected and VP2-vaccinated trout. The fold change in expression observed at 45 days post-challenge in the vaccinated fish relative to the virus carrier fish indicated significant down-regulation. The expression of IFN-1 and a number of ILs was recently studied during acute and persistent infection in Atlantic salmon (Reyes-Cerpa et al., 2012). IL10 is a potent anti-inflammatory cytokine in mammals, and IL8 is a chemokine that induces endothelial activation for the recruitment of immunocompetent cells. The increased expression of IL10 was accompanied by failure to induce IL1b and IL8, indicating that IPNV triggers an anti-inflammatory response, which may be one of the mechanisms that establishes persistence.

The results obtained in the present study, although in younger rainbow trout, are consistent with those described for salmon. Indeed, we recorded the largest increase of IL10 in IPNV carrier fish, which exhibited low levels of IL8. An important role of IL10 in establishing the persistence of several animal viruses has been described previously (Brooks et al., 2006; Wilson et al., 2011). Accordingly, the significant down-regulation of IL10 expression in the surviving group of pcDNA-VP2-challenged fish compared with the virus carrier group is noteworthy. It can be speculated that the activity of the VP2 vaccine contributes to the decrease in the viral load of the kidney cells and, thus, to the down-regulation of the anti-inflammatory response.

Another cytokine that was tested here was TNF2, which is an important pro-inflammatory cytokine that is similar to TNFα (Wang et al., 2011) and is induced after viral infection (Poisa-Beiro et al., 2008). The trout that were carriers of IPNV (either vaccinated or infected) did not express high TNF2 levels. This lag in TNF2 expression may indicate no activation of a potential TNF-induced apoptosis pathway; this was also observed in experimentally infected Atlantic salmon (Ellis et al., 2010; McBeath et al., 2007).

MHC-1 was analysed because previous data have demonstrated that this is regulated in most trout at 7 days post-vaccination (Ballesteros et al., 2012a). Here, we found very low relative expression of MHC-1 in the vaccinated and infected fish, while the relative levels were 14-fold higher in the virus group. MHC-I molecules are involved in cell-mediated immunity through participating in antigen processing and presentation after viral infection, and they are important for host anti-viral immunity due to regulating natural killer cell activity (Landis et al., 2008). Based on our results, it appears that at the time of sampling, the expression of MHC-I, IL8 and IL12 was not enhanced by the presence of the vaccine, and their roles in persistence may therefore be exerted earlier or may be related to viral abundance.

We also analysed the transcription of genes related to mucosal immunity, such as IgM and IgT genes. Our results indicate that IgT is expressed in both vaccinated and virus control fish, but more intensely in the virus control carrier fish, which also exhibited higher expression compared with fish at 10 days post-challenge. The differences were statistically significant. The expression of IgM at 10 days post-challenge was increased in both the pcDNA-VP2-challenged and IPNV control fish. It noteworthy that results from several other studies revealed that both IgM and IgT are up-regulated in response to the haemorrhagic septicaemia virus, a rhabdovirus infecting salmonid fish, indicating that at early time points post-infection, both IgM+ and IgT+ cells are involved in the antiviral response (Aquilino et al., 2014). Here, it can be concluded that vaccination does not enhance the expression of IgM and IgT above the levels observed in the virus control carrier group, but rather, that it mimics viral activity and contributes to maintaining these immunoglobulins at appreciable levels in both groups of fish. However, it remains unclear what immune activity is being induced by the virus that persists in surviving fish or by the VP2 gene, which was expressed in several organs of the orally vaccinated fish. Some recent studies have focused on models of IPNV persistence in fish that may be useful in studying the molecular and immunological aspects of persistence and latency. Genes that are involved in direct interactions or in the degradation of viral proteins are up-regulated (Marjara et al., 2011), and the translation of a transcriptional repressor was shown to be inhibited, suggesting involvement in the control of IPNV replication and a role in maintaining a low number of viral particles in persistently infected cells. In our current study, the administered vaccine diminished the relative expression of the viral VP4 gene, which serves as a marker of infection and of the infective virus. Thus, it is tempting to speculate that as the viral load is low in the carrier fish, the immune activity induced by the DNA vaccine may ultimately contribute to the removal of the remaining virions.

Further work will be necessary to define the pathways related to the activity of IFN and other cytokines or to the activity of other relevant genes in long-term carriers of IPNV as well as to investigate the possible enhancement of antiviral responses mediated by vaccines. The availability of new tools, such as microarrays and large-scale sequence analysis, will surely generate abundant information, and such efforts are likely to provide a better understanding of the viral state of carrier fish.

In conclusion, we have demonstrated that both RT-qPCR and flow cytometry assays show remarkable differences in viral detection between vaccinated and non-vaccinated surviving fish. Indeed, no virus could be isolated from the cell cultures inoculated with samples from the pcDNA-VP2-challenged fish. However, the transcription of relevant genes that are related to immunity was detected in all the fish, and, even at different levels, similar expression profiles were observed in the virus control fish and the vaccinated fish. As a DNA vaccine, the expression of the VP2 transgene could explain this similarity and its contribution to maintaining immune responses. Thus, under our experimental conditions, the orally administered pcDNA-VP2 vaccine not only induces great protection against IPNV but also prevents persistent stages in the examined survivors.

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4. Evaluar la respuesta inmune y protección inducida por una vacuna DNA oral frente al virus de la Necrosis Hematopoyética Infecciosa.

4.1 Estrategias de Vacunación

El primer informe de una vacuna de DNA que codifica el gen de la glicoproteína (G) de la IHNV reveló una alta eficacia (Anderson, Mourich, Fahrenkrug, et al. 1996), cuando es administrado por vía intramuscular. Sin embargo, la administración oral de vacunas frente rhabdovirus no ha obtenido buenos resultados (Serge Corbeil, Kurath, and Lapatra 2000).

En el trabajo de tesis se estudió la eficacia e la vacuna pcDNAIRF1A-G frente al IHNV, administrada por vía oral e intramuscular en trucha arco-iris y la respuesta inmune de peces vacunados estudiada a diferentes tiempos de vacunación.

La vacuna pIRF1A-G fue diseñada y desarrollada por Alonso y Leong (2003) (Alonso et al. 2003). El plásmido con el promotor del factor 1A (IRF-1A) regulador de genes de interferon en trucha arco iris codifica el gen de la glicoproteína (G) del IHNV.

La vacuna pIRF1A-G encapsulada con alginato de sodio se administró por vía oral.

Diseño experimental:

Truchas arco iris de aprox. 1.5 g, 4 cm de longitud y 4 meses de edad

- Grupo de peces (n=10) vacunados por vía oral con 10, 20, 50 y 100 µg de pcIRF1A-G recubierta con alginato de sodio "vacunados IRF-G".
- Grupo de peces con pcIRF1A-G [5 µg] por vía intramuscular n=10 "Control".
- Grupo de peces sin tratamiento n=10 Control.
- Grupo de peces sin tratamiento. Control virus IHNV n=10 "Control IHNV"

A los 30 días post-vacunación, todos los grupos de truchas fueron infectados con IHNV ATCC VR714 ($5x10^5 TCID_{50}\ ml^{-1}$) por inmersión durante 3 horas, excepto el grupo de peces sin ningún tratamiento o Mock.

Tiempos de muestreos: 1, 3, 7 y 15 días post-vacunación.

Muestras: Intestino, Riñón anterior, Bazo, Branquias e Intestino posterior "Midgut".

Métodos: Extracción de RNA total utilizando TriZol, síntesis de cDNA y PCR cuantitativa a tiempo real.

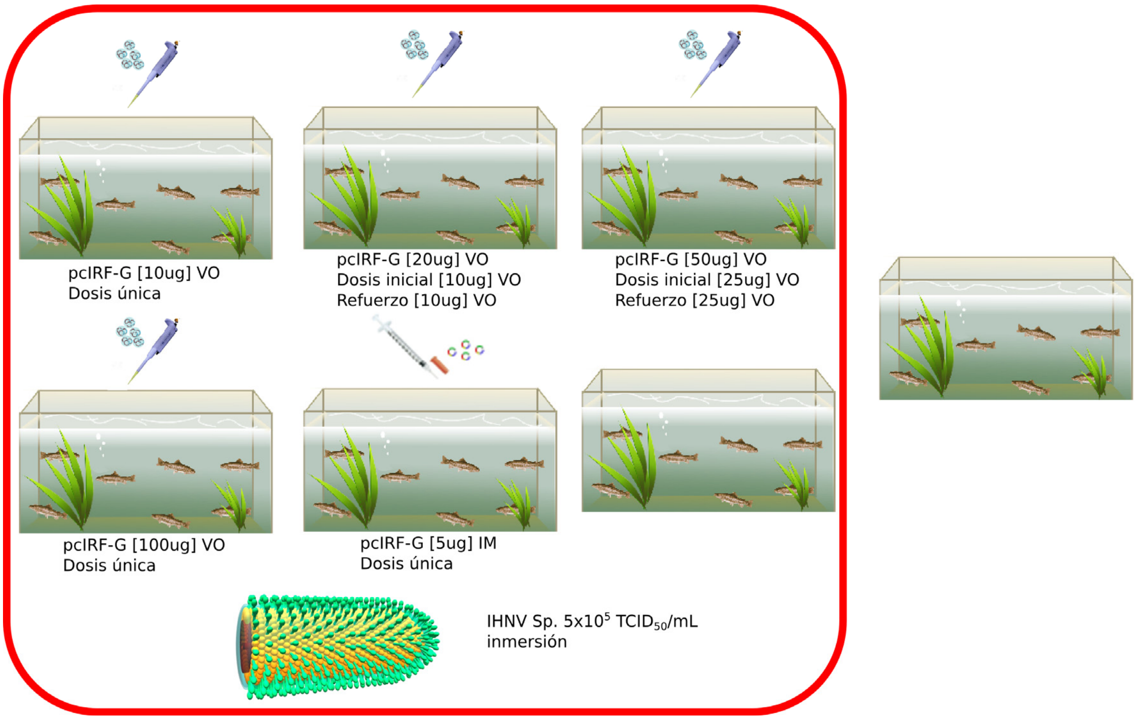

Figura 1: Diseño experimental

Resumen:

Los perfiles de expresión de genes de inmunidad innata más altos se produjeron en el día 3 pv, excepto en branquias en donde el nivel máximo fue a los 7 días. La eficacia de esta vacuna es dosis-dependiente, por lo que los resultados en cuanto a transcripción génica así como en la protección otorgada frente a la infección aumentaron con dosis progresivas de vacuna, obteniéndose los mejores a la dosis de 100 µg por vía oral. Los RPS correspondientes a esta dosis y a la de 5µg por vía IM fueron 56% y 72%, respectivamente. Pese a las diferencias aún considerables en cuanto a eficacia protectora, de nuestros resultados cabe resaltar: a) que hemos demostrado que la vacunación ora frente a IHNV es factible, puesto que logra atravesar el tracto digestivo y ser funcional; b) que pese a que la cepa de virus utilizada es de alta virulencia (100% de mortalidad), se logra protección significativa; y c) que pueden existir vías para su optimización (coadyuvantes moleculares por ejemplo), con lo que se abre un nuevo camino experimental con vacunas “todo pez” más seguras y de fácil administración.

An oral DNA vaccine against infectious haematopoietic necrosis virus (IHNV) encapsulated in alginate microspheres induces dose-dependent immune responses and significant protection in rainbow trout (*Oncorrhynchus mykiss*).

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Administered by intramuscular injection (im.), an all-fish DNA vaccine (pIRF1A-G) containing the promoter regions upstream of the rainbow trout interferon regulatory factor 1A gene (IRF1A) driven the expression of the infectious hematopoietic necrosis virus (IHNV) glycoprotein (G) elicited protective immune responses in rainbow trout (*Onchorrynkuss mykiss*). However, less laborious and cost-effective routes of DNA vaccine delivery are required to vaccinate large numbers of susceptible farmed fish. In this study, the pIRF1A-G DNA vaccine was encapsulated into alginate microspheres and orally administered to rainbow trout. At 1, 3, 5, and 7 d post-vaccination (pv.), IHNV G transcripts were detected by quantitative real-time PCR (RTqPCR) in gills, spleen, kidney and intestinal tissues of vaccinated fish. This result suggested that the encapsulation of pIRF1A-G in alginate microparticles protected the DNA vaccine from degradation in the fish stomach and ensured vaccine early delivery to the hindgut, vaccine passage through the intestinal mucosa and its distribution thought internal and external organs of vaccinated fish. We also observed that the oral route required approximately 20-fold more plasmid DNA than the injection route to induce the expression of significant levels of IHNV G transcripts in kidney and spleen of vaccinated fish. Despite this limitation, increased IFN-1, TLR-7 and IgM gene expression was detected by RTqPCR in kidney of orally vaccinated fish when a 10μg dose of the oral pIRF1A-G vaccine was administered. Remarkably, IFN-1 gene expression was significantly induced at the three assessed time points (3, 7 and 15 d pv.) in kidney of vaccinated fish when compared with unvaccinated fish. In contrast, significant Mx-1, Vig-1, Vig-2, TLR-3 and TLR-8 gene expression was only detected when higher doses of pIRF1A-G were orally administered (50 and 100μg). The pIRF1A-G vaccine also induced the expression of several markers of the adaptive immune response (CD4, CD8, IgM and IgT) in kidney and spleen of immunized fish in a dose-dependent manner. When vaccinated fish were challenged by immersion with live IHNV, evidence of a dose-response effect of the oral vaccine could also be observed. Although the protective effects of the oral pIRF1A-G vaccine after a challenge with IHNV were partial, significant differences in cumulative percent mortalities (CPM) among the orally vaccinated fish and the unvaccinated or empty-plasmid vaccinated fish were observed. Similar levels of protection were obtained after the intramuscular administration of 5μg of pIRF1A-G or after the oral administration of a high dose of pIRF1A-G vaccine (100μg); with 56 and 70 relative percent survival (RPS) values, respectively. When fish were vaccinated with alginate microspheres containing high doses of the pIRF1A-G vaccine (50 or 100μg), a significant increase in the production of anti-IHNV antibodies was detected in serum samples of the vaccinated fish compared with that in unvaccinated fish. At 10 days post-challenge, IHNV N gene expression was nearly undetectable in kidney and spleen of orally vaccinated fish which suggested that the vaccine effectively reduced the amount of virus in tissues of vaccinated fish that survived the challenge. In summary, our results demonstrated a significant increase in fish immune responses and resistance to an IHNV infection after the oral administration of increasing concentrations of a DNA vaccine against IHNV encapsulated into alginate microspheres.

I. INTRODUCTION

Aquaculture is the fastest growing food-production sector in the world, providing a significant supplement to, and substitute for, wild aquatic organisms. However, viral diseases are a primary constraint to the growth of many aquaculture species. Infectious Hematopoietic Necrosis virus (IHNV) is, in large part, responsible for important losses in the salmonid farming industry worldwide. Economic losses are due not only to fish mortality but also to the quarantine, restriction of movement or destruction of IHNV-infected fish stocks. IHNV is a non-segmented, enveloped, single-stranded, negative-sense RNA virus belonging to the genus Novirhabdovirus in the family Rhabdoviridae. The genome of the virus (11 Kb) contains six open reading frames in the following order: nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-structural viral protein (NV), and polymerase genes (L) (Kurath and Leong, 1985; Morzunov *et. al.*, 1995). Previous studies demonstrated that the IHNV G protein is the only viral protein capable of inducing a neutralizing antibody response to IHNV (Engelking *et. al.*, 1989). The virus was first detected in the Pacific Northwest in

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the USA and is considered endemic in this area. Despite regulatory controls to prevent its dissemination, the virus has extended its geographical reach to European and Asiatic countries, usually by the movement of infected eggs or fish. Following a disease outbreak, surviving fish may be asymptomatic carriers for life. Preventive measures, such as effective vaccines, are critical for a sustainable development of the aquaculture industry and have been the focus of extensive research. In addition to reducing the severity of disease losses, vaccines also reduce the need for antibiotics, leave no residues in the environment and do not induce pathogen resistance. Although different types of vaccines against fish viral diseases have been described, DNA vaccines have proven particularly efficacious. In fact, effective DNA vaccination of a large number of fish species against a variety of viral diseases has been demonstrated (Evensen and Leong, 2013; Hlvold *et. al.*, 2014) The first demonstration that the intramuscular injection (im.) of a plasmid DNA encoding the IHNV G gene into fish resulted in the transient expression of the encoded gene and in the generation of protective immunity against an IHNV challenge was reported by Anderson and co workers. This DNA vaccine was designed to express the IHNV G gene under the control of the cytomegalovirus immediate early promoter (CMVIEP). Later, a DNA vaccine against IHNV containing the CMV promoter in place of the CMVIEP promoter was patented in Canada and approved by commercialization in July 2005 by the Canadian Food Inspection Agency. However, the insertion of a promoter form a human pathogen (i.e. the CMV promoter), makes DNA vaccines containing this promoter unsafe for some countries licensing agencies. Therefore, DNA plasmid vectors containing rainbow trout specific promoters to take the place of the human CMV promoter were constructed (Alonso *et. al.*, 2003). One of these all-fish expression vectors, pIRF1A-G, contained the IHNV G gene linked to the promoter region upstream of the rainbow trout interferon regulatory factor 1A (IRF1A). Administered by im., the effectiveness of pIRF1A-G as IHNV DNA vaccine compared favourably with that pCMVIEP-G in vaccine trial. Although DNA vaccines against IHNV and the related fish rhabdovirus, viral hemorrhagic septicaemia virus (VHSV), have been shown to stimulate the production of specific neutralizing antibodies and to induce the expression of non-specific IFN-inducible antiviral genes such as Mx-1 and Vig-1 in rainbow trout (Purcell *et. al.*, 2004), the specific immune mechanisms and correlated of protection of pIRF1A-G vaccinated fish remain to be elucidated. DNA vaccination by im. is very effective in inducing fish immune responses. However, alternative routes of DNA vaccine delivery are desirable for less stressful manipulation of the fish and for use in small fish for which im. is not practical or cost effective. Oral delivery of DNA vaccines is considered the most appropriate way to immunize large numbers of small farmed fish. Advantages of oral vaccine delivery in fish includes its safe, easy application, limited stress effects, and reduced cost, time and labor (Behera and Swain., 2013). However, oral vaccine delivery has some drawbacks mainly due to the strong physiological conditions encountered in the first portions of the fish gastrointestinal tract where very low pH levels may be present (Ellis, 1995). Therefore, some antigen-encapsulation methods have been developed to overcome vaccine degradation in the fish stomach and to ensure the arrival of enough quantity of plasmid vaccine to the second segment of the fish gut where antigen uptake occurs (Salinas *et. al.*, 2011; Spencer *et. al.*, 2013). We have previously conducted several studies using an oral DNA vaccine against infectious pancreatic necrosis virus (IPNV), a member of the *Birnaviridae* family that causes widespread mortality in salmonid fish. The pcDNA-VP2 vaccine, a plasmid vector encoding the VP2 gene of IPNV, was encapsulated into alginate microspheres for oral delivery to rainbow trout (de las Heras *et. al.* 2010). The effectiveness of the pcDNA-VP2 vaccine was demonstrated when the vaccine was orally administered by pipette directly into the mouth of both brown trout (*Salmo trutta*) and rainbow trout (*Onchorrynkuss mykiss*) or incorporated into food pellets. After the oral administration of the vaccine, the VP2 transgene was expressed in several organs of vaccinated fish, induced innate and specific immune responses, and a strong protection against an IPNV challenge. At 30 days post-challenge with live IPNV, relative percent survival (RPS) values of 84 in brown trout and between 67 and 83 in rainbow trout were obtained. By using an oligo microarray, high transcriptional levels of a group of immune-related genes; including IFN-1, Mx-1, Mx-3, IgM and IgT, were detected in kidney of orally vaccinated fish prior challenge suggesting that these genes might be important for protection against IPNV challenges (Ballesteros *et. al.*, 2012). Overall, these studies demonstrated the potential of using alginate microspheres as an effective strategy to deliver DNA vaccines against fish viral diseases and provided some protection correlates that could be used to test efficacy of oral DNA vaccines. In the current study, we compared the protective immune responses generated by two routes of administration of an all-fish IHNV DNA vaccine (pIRF1A-G) including: intramuscular injection and oral delivery in alginate microparticles Expression of IHNV G mRNA transcripts, and mRNA expression profiles of several markers of the innate and adaptative immune responses, including IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7, TLR-8, CD4, CD8, IgM and IgT genes, were examined in kidney and spleen of vaccinated fish, in order to understand the protective mechanisms induced by both delivery routes. In addition, the efficacy of the pIRF1A-G vaccine given orally or by im. in inducing anti-IHNV antibodies and protection against an IHNV challenge was compared. Finally, we evaluated whether the oral administration of pIRF1A-G would effectively reduce viral load in vaccinated fish.

II. MATERIALS AND METHODS

A. Ethics statement

All the in vivo procedures were performed in strict accordance with the recommendations in the European Union Ethical Guidelines for the care of animals used for experimental and other scientific purposes (2010/63/EU). All the experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Consejo Superior de Investigaciones Cientficas (CSIC).

B. Fish rearing conditions.

Healthy rainbow trout specimens (3-4 ± 0.3 g mean weight) were purchased from a spring water farm with no history of viral diseases (Guadalajara, Spain). Fish were maintained under a 12-h- light/12-h-dark photoperiod at 13 ± 1ºC in 350-L closed flow-through water tanks (Living Stream, Frigid Units Inc., Ohio at the Centro de Investigaciones Biolgicas (CSIC, Madrid, Spain) Fish were fed daily (1.5% body weight) with a pelleted diet (Skretting, Spain). A pool of five trout was tested to confirm the absence of IHNV or any other salmonid virus by isolation using BF2 cells (Alonso *et. al.*, 1999). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks, and during this period, no clinical signs were observed.

C. Encapsulation of the pIRF1A-G DNA vaccine in alginate microspheres

The pIRF1A-G DNA vaccine, which contains the promoter regions of the rainbow trout interferon regulatory 1A gene driven the expression of the IHNV glycoprotein gene was kindly provided by Dr. Marta Alonso and Dr. Jo-Ann Leong. The pIRF1A-G vaccine is covered by a patent application through Oregon State University (WO200269840 and Alonso and Leong, 2012. Construction of the pIRF1A-G vaccine was previously reported (Alonso *et. al.*, 2003). An empty plasmid lacking the IHNV G gene (pIRF1A) was used as plasmid control in the immunization experiments. Large-scale preparations of the pIRF1A-G vaccine and pIRF1A empty plasmid were prepared from lysates of Escherichia coli TOP10 super competent cells grown in presence of ampicillin (Life Technologies, Alcobendas, Spain). The plasmid DNAs were purified with the Endofree Plasmid Maxi purification kit according to the manufacturers instructions (Qiagen Iberia, S. L. Spain). The DNA concentration was measured in a spectrophotometer before it was aliquoted and conserved at -20ºC. Purified pIRF1A-G DNA vaccine and pIRF1A empty plasmid (1.5 mg) were encapsulated into alginate microspheres as previously described. Briefly, 2.5 ml of 3% (w/v) of sodium alginate were mixed with 1.5 ml of 1 mg/ml of pIRF1A-G or pIRF1A and the mixture stirred at 500 rpm during 10 min. This solution was added to an Erlenmeyer flask containing 100 ml of paraffin oil and 0.5 ml Span 80, and the mixture was emulsified for 30 min at 900 rpm. Microspheres were prepared by adding 2.5 ml of 0.15 M $CaCl_2$ to the emulsion drop by drop and stirring for 2 h at 900 rpm. Microspheres were collected by centrifugation at 1000 g for 10 min, and they were washed twice with 70% ethanol, lyophilized for 24 h and stored at 4ºC. The DNA content of the pIRF1A-G-loaded alginate microspheres was assessed by resuspending plasmid-loaded alginate microspheres in 5 ml of sodium citrate (55 mM) overnight at room temperature. The absorbance of the supernatant was measured at 260 nM in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

D. Immunization trials

In the first laboratory trial six experimental groups of 50 fish each (mean weight 3-4 g) were compared: (1) fish were orally immunized with a suspension of alginate microparticles containing 10μg of pIRF1A-G vaccine, (2) fish orally immunized with 10μg of pIRF1A-G in alginate microparticles and boosted 15 days later with the same amount of plasmid (3) fish orally immunized with alginate microparticles containing 25μg of pIRF1A-G, (4) fish orally vaccinated with 25μg of pIRF1A-G in alginate microparticles and boosted 15 days later with the same amount of DNA, (5) fish orally immunized with alginate microparticles containing 10μg of pIRF1A empty plasmid, and (6) control, unvaccinated fish. For oral immunization, fish were anaesthetised by immersion in 50μg/ml of buffered tricaine methane sulphonate (MS-222; Sigma-Aldrich, Madrid, Spain) and then the corresponding amount of plasmid-loaded microspheres was introduced into the mouth of each fish with an automatic pipette supporting a 20μl tip end at the entrance of the esophagus. In the second experimental trial, four experimental groups of fish (n = 50) were compared, including: (1) fish orally immunized with alginate microparticles containing 100μg of pIRF1A-G, (2) fish injected at the base of the dorsal fin with 5μg of pIRF1A-G, (3) fish orally immunized with alginate microparticles containing 100μg of pIRF1A empty plasmid, and (4) control, unvaccinated fish.

E. Tissue collection, RNA extraction, cDNA synthesis and quantification of gene expression by a two-step quantitative reverse-transcription PCR (RTqPCR)

Fish were sacrificed via MS-222 overdose. Gills, head kidney, spleen, and/or intestinal tissues were aseptically collected from vaccinated and unvaccinated trout and individually stored in 1 ml of TRI-

Code	Name	Abbreviation	Primer Sequence (5′-3)
NM_001124531	Type I Interferon	IFN-1	F/AAAACTGTTTGATGGGAATATGAAA R/CGTTTCAGTCTCCTCTCAGGTT
NM_001171901	Interferon-induced protein Mx1	Mx-1	F/AGCTCAAACGCCTGATGAAG R/ACCCCACTGAAACACACCTG
U47946.1	Interferon-induced protein Mx3	Mx-3	F/AGCTCAAACGCCTGATGAAG R/TGAATATGTCTGTTATCCTCCCAAA
X65263.1	Membrane bound Immunoglobulin M	IgM	F/ACCTTAACCAGCCGAAAGGG R/TGTCCCATTGCTCCAGTCC
AY870265	Immonoglobulon Tau heavy chain	IgT	F/AGCACCAGGGTGAAACCA R/GCGGTGGGTTCAGAGTCA
AF498320	Elongation factor EF1 alpha	EF-1^{α}	F/GATCCAGAAGGAGGTCACCA R/TTACGTTCGACCTTCCATCC
	N Gene	N-IHNV	F/TGTGCATGAAGTCAGTGGTGG R/CCTGCTCATCATGACACCGTA
	G Gene	G-IHNV	F/GCGCACGCCGAGATAATATCAA R/TCCCGTGATAGATGGAGCCTTT P/CGATCTCCACATCCCGGAATAAATGACGTCT
		β-ACTIN	F/GGCCGTGTTGTCCCTGTAC R/CCGGAGTCCATGACGATACC P/CCTCTGGCCGTACCACC

TABLE I: Genes and primer sequences used in the RTqPCR assays.

zol Reagent (Invitrogen, Spain) at -70°C until RNA isolation. Tissue samples were homogenized using the Tissue Lyser Cell Disruptor (Qiagen S. A., Madrid, Spain) 5 min at 50 Hz with 2 mm glass beads. Total RNA was isolated from different tissues by using the TRIzol LS Reagent according to the manufacturers instructions. RNAs were treated with DNase I RNase Free (Fermentas, Spain) to remove genomic DNA that might interfere with the PCRs. The purity and the yield of the RNA samples were analysed in a NanoDropTM 1000 spectrophotometer. RNA quality was determined by measuring the 260/280-nm absorbance ratio, and ratios of 1.8 or higher were considered acceptable for purified RNA. Total RNA (5μg) was then reverse transcribed to c-DNA using the Super ScriptTM III cDNA synthesis kit and oligo-(dT) primer (25 pmol/μl) according to the manufacturers instructions (Life Tecnologies). The cDNA was diluted 1:4 in DEPC treated water and 2μl of the diluted cDNA was used in each real-time qPCR reaction. Real-time qPCR reactions for the amplification of each target gene were performed in a 25μL final volume containing 12.5μL of 2 x Quantimix Easy SYBR Green (Biotools Labs, Madrid, Spain), 0.3μM of forward and reverse primers, 8.5μL of ultrapure water and 2μL of diluted cDNA. Real-time qPCR amplifications of c-DNA were accomplished in an iQ5 iCycler Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Madrid, Spain) under the following conditions: 1 cycle of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30s, annealing at 60°C for 1 min, and a dissociation cycle (1 min at 95°C and 1 min at 60°C). After the run, the melting curve of each amplicon was analysed to determine the specificity of the amplification. Table 1 shows the list of the amplified trout genes, their accession numbers and corresponding primer sequences. Since the trout Elongation Factor 1α (EF-1α) is constitutively expressed, it was used as the endogenous gene control to normalize the expression of each target gene in each RNA sample. Results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta \mathrm{CT}}$ where ΔCT is determined by subtracting the EF-1α threshold cycle (CT) from the target CT value. Negative controls with no template where included in all experiments. All the PCR amplifications were performed by duplicate, and each experiment was repeated twice to confirm the results.

F. IHNV experimental challenge by immersion

At 30 days after vaccination, duplicate sets of 15 fish (average weight of 4 g) from each treatment group were challenged by immersion with 10^5 TCID_{50}/ml of live IHNV (ATCC VR 714) for 3 h at 14 $\pm$ 1°C with aeration. Dead fish were collected daily for 30 days post-challenge. Moribund fish displayed classical signs of IHNV infection such as distended abdomens, darkened body coloration and vertical drifting. Approximately, 10% of the dead fish were assessed by PCR amplification of the IHNV N gene to confirm that they died as a result of an IHNV infection. Efficacy of the vaccines was assessed by comparing the final CPM among vaccinated and unvaccinated fish. The relative percent survival (RPS) value of each experimental group was calculated according to the following formula: RPS = [1 - (CPM vaccinated fish/CPM unvaccinated fish] × 100 (Jarp and Tverdal., 1997).

G. Indirect enzyme-linked immunosorbent assay (ELISA) for the detection of anti-IHNV serum antibodies

At 15, 30 and 45 days pv., rainbow trout were sacrificed by immersion in an overdose solution of MS-222 (200μg /ml), and blood was collected from the caudal vein of vaccinated and unvaccinated fish using a 25-gauge needle (Becton-Dickinson) Blood samples were allowed to clot at room temperature for 2 h, stored at 4°C overnight, and then centrifuged at 500 g for 10 min. Serum samples were collected and stored at -20°C until they were analysed by ELISA. Briefly, 96-well microtiter plates (Greiner Bio-one, Frickenhausen, Germany) were coated with 100μL IHNV previously propagated in BF-2 cells; the cell culture medium was used as negative antigen control. After incubation for 18 h at 4°C, unbound virus was removed by washing each well five times with 200μl of PBS with 0.05% Tween 20 (PBS-T). The plates were then blocked for 2 h at 22°C with blocking solution consisting of 5% skimmed milk in PBS. The plates were washed three times with PBS-T, and 100μl of each fish serum sample serially diluted in PBS with 1% BSA was added in triplicate. After 3 h of incubation at 22C, the plates were washed three times with 200 μl of PBS-T. Each well was then inoculated with 100μL of the anti-rainbow trout IgM monoclonal antibody (Aquatic Diagnostics Ltd, Stirling, Scotland) and incubated at room temperature for 1 h. Unbound antibody was removed after three washes with 200μl of PBS-T Each well was then inoculated with 100μl of horseradish peroxidase-conjugated goat anti-mouse IgM polyclonal antibody (Dako, Glostrup, Denmark), diluted 1:1,000 in PBS with 1% BSA and incubated at 22°C for 1h. Unbound conjugate was removed after 5 washes with 200μl of PBS-T, and then antibody binding was visualized by adding 100μL of 3, 3, 5, 5-tetramethylbenzidine dihydrochloride (TMB) to each well. The plates were incubated for 10 min at 22°C in the dark, and the reactions were stopped by adding 50μl of H2O2 per well. The optical density (OD) in each well was measured at 450 nm by an ELISA plate reader (Bio-Rad). The specific antibody titre of each sample was expressed as mean OD values (± SE) after subtracting OD values from the control unvaccinated fish.

H. Statistical analysis

Differences in CPMs between vaccinated and control fish groups were statistically analysed, using a Fishers exact test (GraphPad Prism version 4.03, GraphPad Software, San Diego, CA). Differences in gene expression between experimental groups were analysed using factorial ANOVAs with the Tukey-Kramer adjustment for multiple comparisons (IBM SPSS Statistics 15; Chicago, IL, US). In all analyses, differences between groups were considered statistically significant when the correlation value P was $<$ 0.05

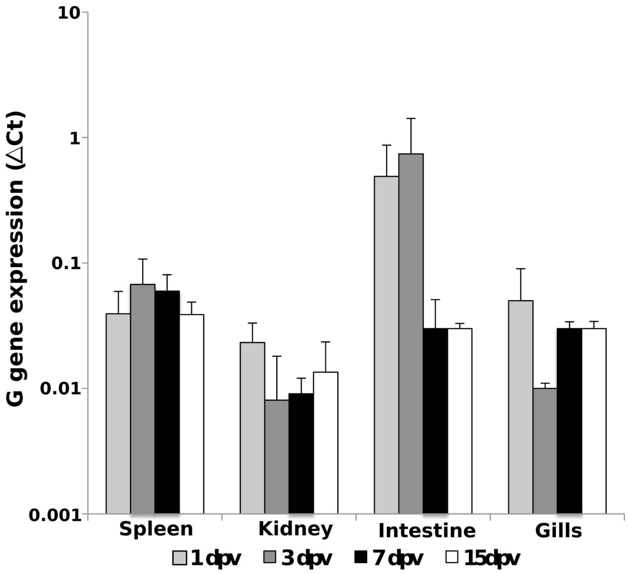

FIG. 1: IHNV G gene expression in trout tissues after oral immunization with pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10μg of alginate- encapsulated pIRF1A-G vaccine. IHNV G expression was analysed by RTqPCR in gills, kidney, spleen and intestinal tissues collected from four vaccinated rainbow trout 1, 3, 7 and 15 days after inmunization Results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as the mean $2^{-\Delta CT}$ where ΔCT is the C_t (target gene) - C_t (EF-1α). Bars represent the mean (± SD).

III. RESULTS

A. HNV G transcripts were expressed in several fish organs early after oral vaccination with pIRF1A-G encapsulated in alginate microspheres

To verify whether alginate microspheres protected the pIRF1A-GDNA vaccine from stomach degradation, IHNV G expression was examined in gills, kidney, spleen, and intestinal tissues of rainbow trout vaccinated with a 10μg dose of the vaccine. At 1, 3, 7 and 15 days pv., low but appreciable levels of transgene expression were observed in all the examined organs (Fig 1). When the G gene expression in the four examined organs was compared, the lowest expression levels were detected in kidney of vaccinated fish at the four assessed time points. In contrast, the highest IHNV G gene expression levels were recorded in intestinal tissues of the vaccinated fish at 1 and 3 days pv. These findings suggested that the encapsulation of pIRF1A-G vaccine in alginate microspheres ensured the early delivery of the vaccine to the hindgut, its passage through the intestinal mucosa and its distribution thought internal and external organs of vaccinated fish.

B. Expression of IHNV-G, TLRs, IFN-1, and IFN-inducible genes in response to the oral or intramuscular administration of pIRF1A-G vaccine

We compared the transcriptional profiles of several host immune responses markers after the oral pIRF1A-G immunization with those induced after pIRF1A-G injection. Some genes previously found highly expressed (>2-fold) in oral pcDNA-VP2 vaccinated trout and/or induced after IPNV-infection were selected for the current analysis (Ballesteros *et. al.*, 2012). The selected genes belonged to (i) the innate immune response such as IFN-1 (Robertsen, 2008), Mx-1 (Trobridge *et. al.*, 1997), Mx-3 (Trobridge *et. al.*, 1997), Vig-1 (Boudinot *et. al.*, 1999), Vig-2 (Boudinot *et. al.*, 2001), TLR-3 (Rodriguez *et. al.*, 2005), TLR-7, TLR-8 (Palti *et. al.*, 2010); and (ii) the adaptative immune response such as IgM (Lorenzen *et. al.*, 1993), IgT (Zhang *et. al.*, 2010), CD4 (Castro *et. al.*, 2014) and CD8. Table 1 shows the corresponding primer sequences designed for the RTqPCR analysis of those genes. Because head kidney and spleen are both target organs of IHNV multiplication and two of the most important trout immune-responsive organs, they were both selected as target internal organs for transcriptional analysis. In order to determine whether increasing doses of the oral pIRF1A-G vaccine would significantly increase the expression of the selected immune markers groups of rainbow trout were immunized with alginate microparticles containing 10, 25 or 100μg of pIRF1A-G-loaded alginate microparticles. Fish intramuscularly injected with 5μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. At 3, 7 and 15 days pv., kidney and spleen were collected from four fish of each group and analysed for gene expression by RTqPCR. The transcription levels of the rainbow trout Mx-1, Mx-3, Vig 1, Vig 2, TLR-3, TLR-7 and TLR-8 genes, in kidney and spleen of vaccinated and unvaccinated animals were normalised to the expression of the endogenous control, EF-1α. and the corresponding results are shown in Figures 2 and 3 according to the assessed organ. The expression of the IHNV G gene was also included in the analysis as a vaccine marker. As expected, our results demonstrated that the expression of the IHNV G transgene in kidney and spleen of vaccinated animals increased with increasing concentrations of oral DNA vaccine Of the three assessed doses of oral vaccine, only the 100μg dose induced significant levels of IHNV G transcripts in kidney and spleen of vaccinated fish. Furthermore, higher levels of IHNV G expression were detected in kidney of fish orally vaccinated with 100μg of pIRF1A-G vaccine than in injected fish at all the assessed time points. Only at day 7 pv., higher levels of IHNV G expression were detected in spleen of fish orally vaccinated with the 100μg dose when compared with the injected fish.

Gene expressionanalysis inkidney of oral and intramuscular vaccinated fish showed significant increases in the expression of most of the examined host genes, when compared with unvaccinated fish (Fig 2). After oral vaccination increased IFN-1 and TLR-7 gene expression was observed in kidney of vaccinated fish even when the lowest dose of the vaccine was administered. Statistical analysis of the date indicated that theMx-1, Vig-2, and TLR-3 genes were highly expressed in fish orally vaccinated with a vaccine dose $\leq$ 25μg. When compared with unvaccinated fish, a significant increase inVig-1, Mx-3 and TLR-8 gene expression was only detected in kidneys of the fish orally vaccinated with the highest dose of the DNA vaccine (100μg). Remarkably, a significant induction of IFN-1 gene expression was detected after immunization with the three assessed doses of oral DNA vaccine and at 3, 7 and 15 days pv. when compared with unvaccinated fish. When we compared the two routes of vaccine delivery, we detected similar IFN-1 gene expression in kidney of injected fish and in the fish groups that were vaccinated with each dose of the oral DNA vaccine. However, significant differences in the expression of the Mx-1 at day 3 pv., Mx-3 at day 7 pv. and TLR-7 and TLR-8 genes at days 7 and 15 pv. were observed depending on the vaccine delivery method. Interestingly, the administration of 100μg of the oral vaccine induced high levels of TLR-7 and TLR-8 gene expression over time that at 7 and 15 days after the vaccination were higher than the levels induced by the intramuscular injection of the DNA vaccine, while no differences were recorded in the first 3 days pv. As seen in figure 2, the increase in TLR-7 and TLR-8 gene expression 3 days after the intramuscular administration of the vaccine was an initial response which was then quickly suppressed.

The transcription levels of the rainbow trout Mx-1, Mx-3, Vig 1, Vig 2, TLR-3, TLR-7 and TLR-8 genes were also examined in the spleens of the fish orally vaccinated with increasing concentrations of oral pIRF1A-G vaccine. The corresponding results are presented in Figure 3. As expected, a dose-dependent effect of the vaccine on gene expression was evident. When gene expression in spleen of fish injected with 5μg of pIRF1A-G and orally vaccinated with 100μg of the vaccine was compared, similar levels of IFN-1, Vig-2 and Mx-1 expression were detected at three assessed time points. Remarkably, the administration of 100μg of the oral vaccine induced higher levels of TLR-3, TLR-7 and TLR-8 gene expression in spleen of the vaccinated animals at day 7 pv. than did the intramuscular injection of the DNA vaccine. At 15 days pv., only the TLR-8 gene remained highly expressed in spleen of orally vaccinated fish when compared with the levels of expression of this gene in im. fish.

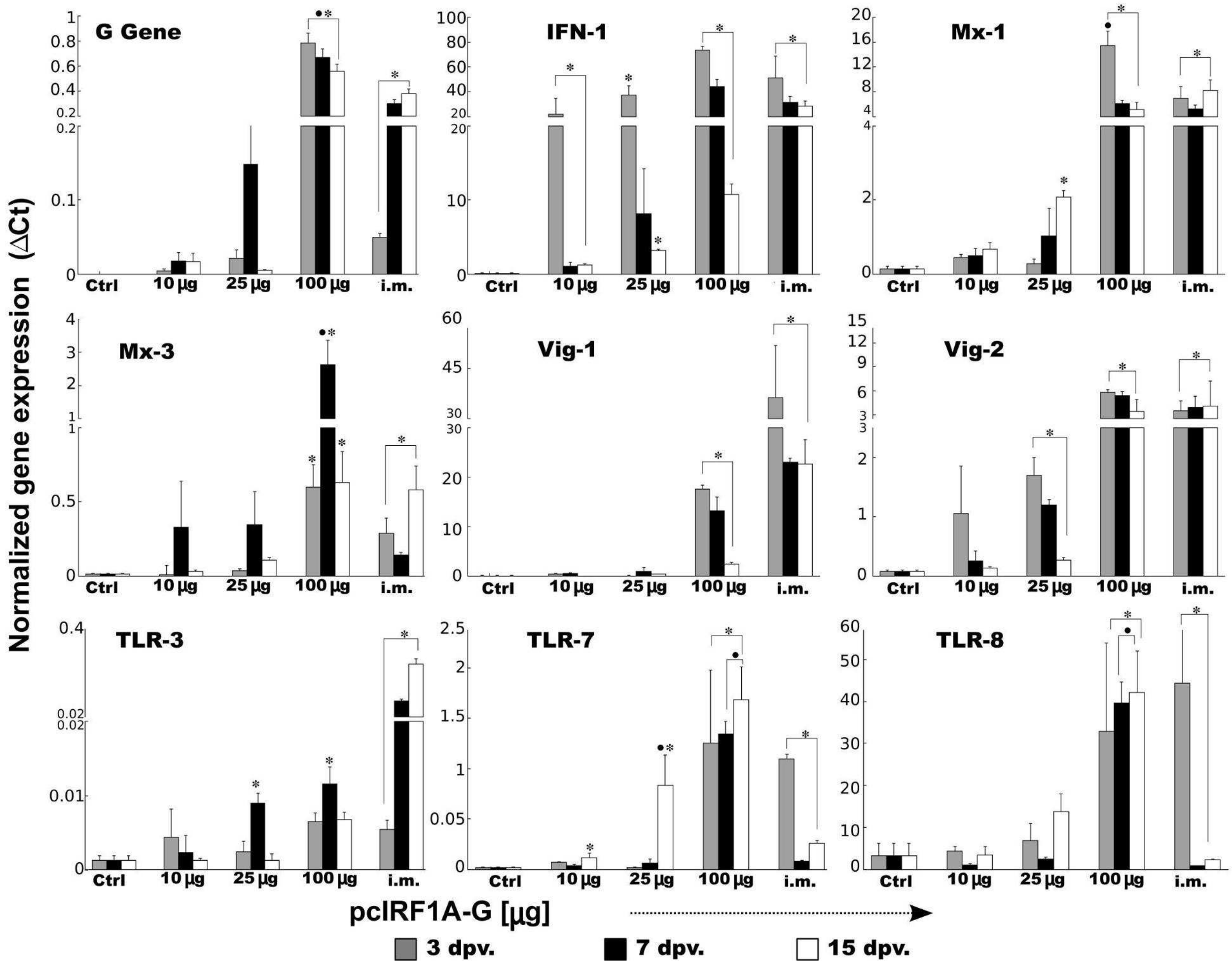

FIG. 2: IHNV-G, TLRs, IFN-1, and IFN-stimulated gene expression in trout kidney after oral immunization with increasing concentrations of pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10, 25 and 100μg of alginate- encapsulated pIRF1A-G vaccine. IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7, and TLR-8 gene expression was evaluated by RTqPCR in kidney of four vaccinated fish at 1, 3, 7 and 15 d pv. The analysis of the IHNV G expression was also included as a vaccine marker. Fish intramuscularly injected with 5μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. RTqPCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta \mathrm{CT}}$ where ΔCT is the mean C_t (target gene) - C_t (EF-1α). Asterisks and black points indicate significant differences ($P \leq 0.05$) between vaccinated and unvaccinated fish or between oral and intramuscular vaccinated fish, respectively.

C. CD4, CD8, IgM and IgT gene expression in response to the oral or intramuscular administration of the pIRF1A-G vaccine

Cellular-specific immune responses induced after the oral or intramuscular administration of pIRF1A-G vaccine were evaluated at 15 days pv. in spleen and kidney of vaccinated fish by RTqPCR. Four markers of the cellular immune responses including the rainbow trout CD4, CD8, IgM and IgT genes were selected for gene expression analysis. As seen in Figure 4, CD4 and CD8 expression levels were significantly higher in kidney of orally vaccinated fish (100μg dose) than in injected fish. A lower dose of the oral vaccine (25μg) was able to induce significant levels of CD4 gene expression in spleen of vaccinated fish. CD8 expression levels were lower than those of CD4 regardless of the treatment and were higher in the fish vaccinated with 100μg of pIRF1A-G than in any other experimental group. Interestingly, the im. of the vaccine did not induce significant levels of CD8 gene expression in kidney and spleen of vaccinated fish when compared with unvaccinated fish.

The transcription levels of IgM and IgT genes at 15 days pv. were also analysed by RTqPCR in spleen and kidney of intramuscularly and orally vaccinated fish (Fig 4). No significant differences in IgM gene expression were observed between the injected and orally vaccinated fish regardless of the assessed tissue. When compared with unvaccinated fish, administration of the vaccine orally or by injection induced significant IgM expression in kidney tissues. Even at the lowest dose of the oral vaccine, a significant increase in IgM expression was detected in the kidney of

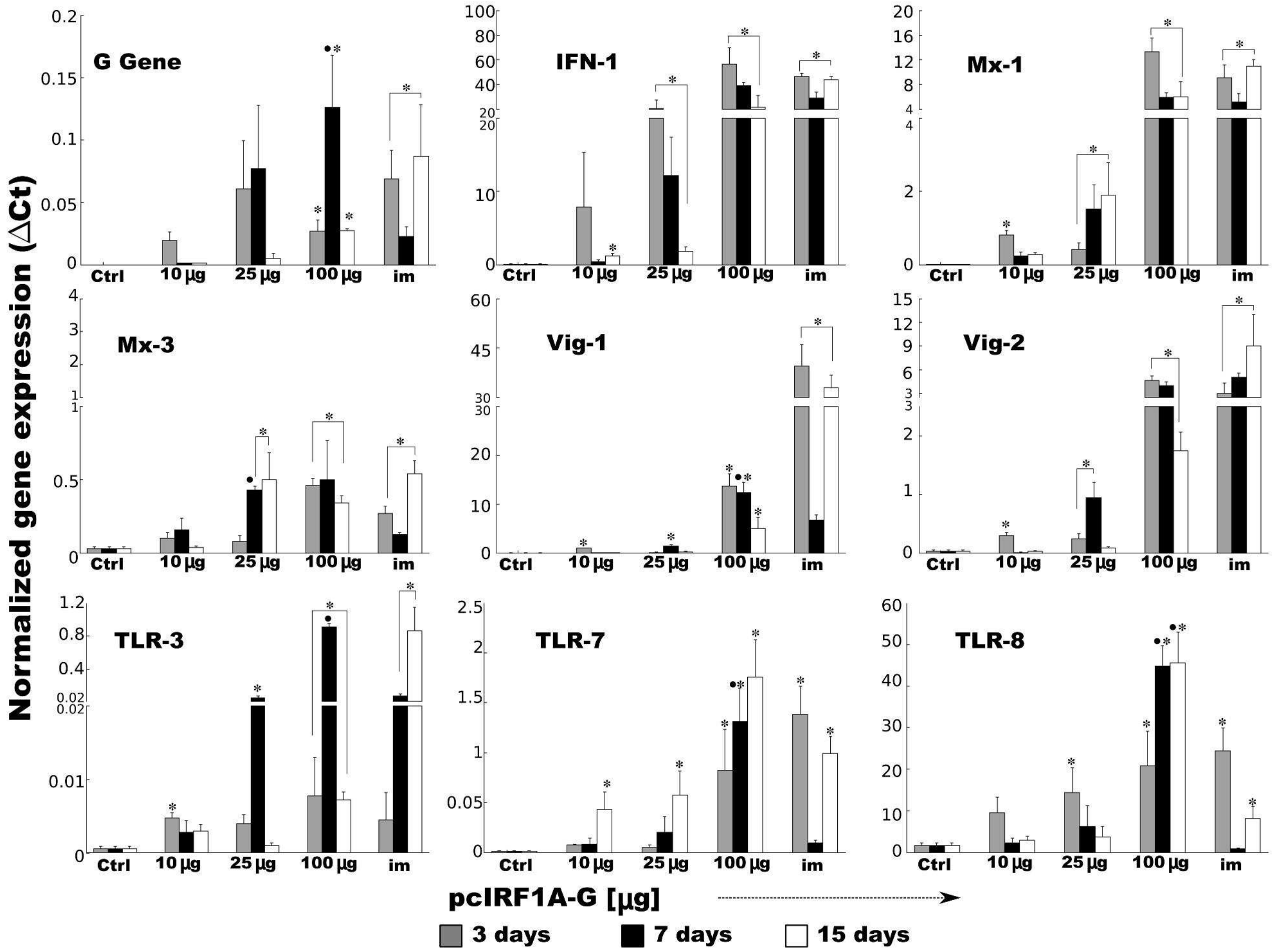

FIG. 3: IHNV-G, TLRs, IFN-1, and IFN-stimulated gene expression in trout spleen after immunization with increasing concentrations of pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10, 25 and 100μg of pIRF1A-G-loaded alginate microparticles. IFN-1, Mx-1, Mx-3, Vig-1, Vig-2, TLR-3, TLR-7, and TLR-8 gene expression was evaluated at 1, 3, 7 and 15 d pv. by RTqPCR in spleen of four vaccinated fish. The analysis of the expression of the IHNV G gene was also included as a vaccine marker. Fish intramuscularly injected with 5μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. RTqPCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔCT is the mean C_t (target gene) - C_t (EF-1α). Asterisks and black points indicate significant differences ($P \leq 0.05$) between vaccinated and unvaccinated fish or between oral and intramuscular vaccinated fish, respectively.

vaccinated animals when compared with unvaccinated fish. In contrast, only the highest dose of the oral vaccine induced significant IgT expression in spleen of vaccinated animals when compared with unvaccinated fish. Remarkably, no significant production of IgT transcripts were detected in kidney of the im. fish.

D. Alginate-encapsulated pIRF1A-G vaccine protected orally vaccinated fish against a lethal challenge with IHNV

In the first experimental trial, increasing concentration of alginate-encapsulated pIRF1A-G vaccine were orally administered to rainbow trout fry and evaluated for their efficacy in inducing a protective immune response against an IHNV challenge. Groups of 50 rainbow trout were orally immunized with alginate microparticles containing (1) 10μg of pIRF1A-G, (2) 10μg of pIRF1A-G and boosted 15 days later with the same amount of plasmid, (3) 25μg of pIRF1A-G, and (4) 25μg of pIRF1A-G and boosted 15 days later with the same amount of DNA. Fish orally immunized with alginate microparticles containing 10μg of empty-plasmid and unvaccinated fish were used as negative controls. At 30 days pv., two subgroups of 15 fish form each group were challenged by immersion with 10^5 $TCID_{50}$ /ml of IHNV. Mortality was recorded daily for 30 days. The effects of increasing concentrations of the oral DNA vaccine on CPM are shown in Figure 5A. In general, our results showed that alginate-encapsulated pIRF1A-G vaccinated fish exhibited less mortality than the empty-plasmid vaccinated or unvaccinated fish. Oral immunization with 10, 20, 25 and 50μg of the oral DNA vaccine resulted

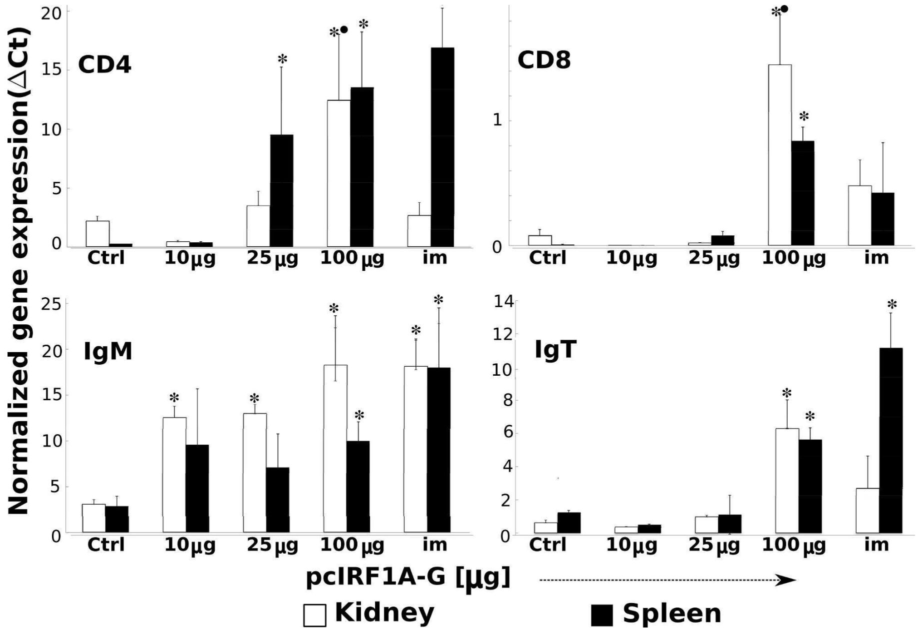

FIG. 4: CD4, CD8, IgM and IgT gene expression in trout kidneys and spleens after oral immunized with pIRF1A-G-loaded alginate microparticles. Rainbow trout were immunized with 10, 25 and 100μg of pIRF1A-G-loaded alginate microparticles. CD4, CD8, IgM and IgT gene expression was evaluated at 1, 3, 7 and 15 d pv. in kidney and spleen of four vaccinated fish. Fish intramuscularly injected with 5μg of pIR1A-G and unvaccinated fish were used as positive and negative controls, respectively. RTqPCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔCT is the mean C_t (target gene) - C_t (EF-1α). Asterisks and black points indicate significant differences ($P \leq 0.05$) between vaccinated and unvaccinated fish or between oral and intramuscular vaccinated fish, respectively.

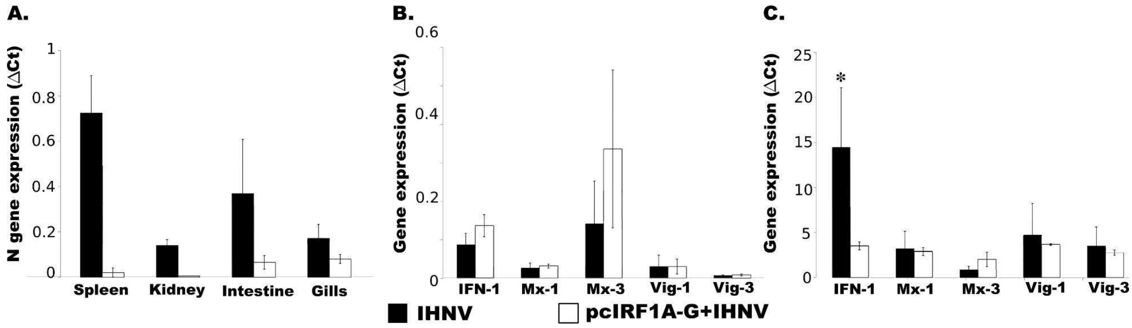

FIG. 5: A- Cumulative percent mortalities (CPM) of rainbow trout after the oral administration of the alginate-encapsulated pIRF1A-G vaccine and subsequent challenge with IHNV. A- Groups of 50 rainbow trout were orally immunized with alginate microparticles containing 10μg of pIRF1A-G, 10μg of pIRF1A-G and boosted 15 days later with the same amount of plasmid, 25μg of pIRF1A-G, and 25μg of pIRF1A-G and boosted 15 days later with the same amount of DNA. Fish orally immunized with alginate microparticles containing 10μg of empty-plasmid and unvaccinated fish were used as negative controls. At 30 days pv., two subgroups of 15 fish form each group were challenged by immersion with IHNV at a concentration of 10^5 $TCID_{50}$/ml. Mortality was recorded daily for 30 days. B- In a second trial, groups of 50 trout each were immunised by intramuscular injection with 5μg of pIRF1A-G or orally with 100μg of pIRF1A-G-loaded alginate microparticles. As controls, groups of fish received the empty-plasmid orally or by im. After 30 days, the fish were challenged with IHNV (1×10^5 $TCID_{50}$/ml) by immersion and monitored for the next 30 days. CPM of each treatment group was recorded, and the relative percent survival (RPS) was calculated using the formula RPS= [1 - (% mortality vaccinated fish/% mortality control fish) - 100]). Two replicates of the trial were performed.

in CMPs at 30 days post-challenge of 78, 70, 70, and 54%, respectively. In contrast, the control fish that received orally the empty-plasmid and the unvaccinated animals exhibited CMPs of 90 and 100%, respectively. Although the protective effects of the oral pIRF1A-G vaccine after challenge with live IHNV were partial, statistically significant differences in mortalities between the fish orally vaccinated with 10, 20, 25 and 50μg of the DNA vaccine and the empty-plasmid vaccinated or unvaccinated fish were observed. No significant differences in mortalities were observed between the empty-plasmid vaccinated fish and the unvaccinated fish ($P = 0.0015$). CMP in the challenged pIRF1A-G (50μg) group was 54% and 100% in the unvaccinated group, resulting in an RPS of 45. In the second experimental trial, the effect of a higher dose of the oral pIRF1A-G vaccine (100μg) on fish CPM was evaluated. Fish injected with 5μg of pIRF1A-G and orally vaccinated with the empty plasmid were used as positive and negative control groups, respectively. Unvaccinated fish were used as the negative control group for RPS calculations. As shown in figure 5B, immunization with 100μg of the oral pIRF1A-G vaccine resulted in a CPM of 43.75 while the CPM in the injected group was 30% (P= 0.0566). In contrast, 100% and 90% of the unvaccinated and empty-plasmid vaccinated fish succumbed to virus challenge, respectively. Therefore, injected fish and orally vaccinated fish were significantly protected against the challenge with IHNV when compared with unvaccinated or empty-plasmid injected fish ($P \leq 0.001$). RPS values were calculated for each challenged experimental group. Our data indicated that the oral administration of a 100μg dose of pIRF1A-G induced significant protection similar to that obtained when fish were vaccinated by im. with 5μg of the DNA vaccine; 56 and 70 RPS, respectively.

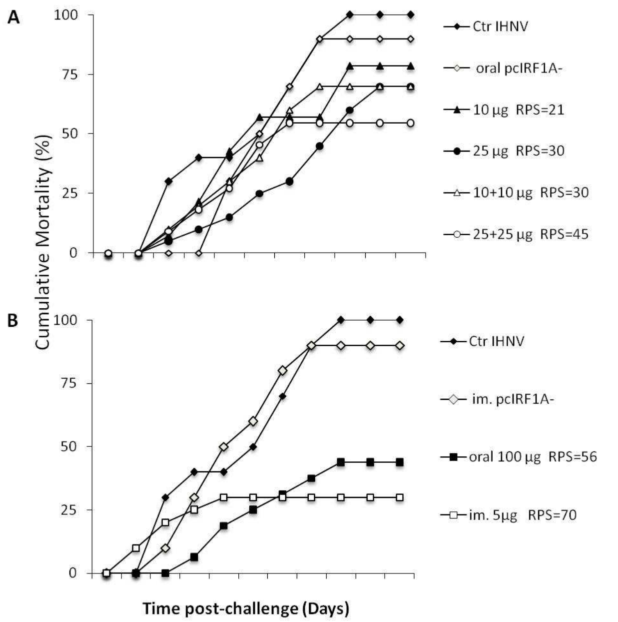

FIG. 6: Viral load as measured by the quantification of IHNV N mRNA (A) and cytokine gene expression (B) in tissues of trout orally vaccinated and infected with IHNV. The fish were orally vaccinated with alginate-encapsulated pIRF1A-G plasmid (10μg per fish) or left unvaccinated. At 30 days pv., each fish group was divided into two groups of 10 fish that were either infected with IHNV or mock infected. At 10 days post-challenge, three trout from each group were sacrificed, RNA was individually extracted, and the levels of expression of the IHNV N gene in spleen, head, kidney, intestine, and gills were determined using a real time-relative expression assay. RTqPCR results were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) and expressed as $2^{-\Delta CT}$ where ΔCT is the mean C_t (target gene) - C_t (EF-1α). Values with asterisks are statistically different ($P \leq 0.05$).

E. Assessment of IHNV viral load in vaccinated and unvaccinated trout after challenge with live IHNV

To determine whether the oral administration of pIRF1A-G (10μg dose) would reduce viral load in vaccinated fish, the transcriptional levels of the IHNV nucleoprotein gene were quantified by RTqPCR in spleen, kidney, gills and intestinal tissues of vaccinated and unvaccinated fish that survived the IHNV challenge (figure 6). At 10 days post-challenge, the levels of IHNV N gene expression in vaccinated fish were lower than that quantified in unvaccinated fish regardless of the assessed organ. Furthermore, N transcript expression was highest in spleen of unvaccinated fish than in other tested organs. Conversely, N gene expression was nearly undetectable in kidney and spleens of vaccinated fish which suggested that the oral vaccine significantly reduced the amount of virus in fish internal organs. As consequence, there risk that vaccinated carriers will spread the disease is much lower than unvaccinated carriers.

F. IFN-1, Mx-1, Mx-3, Vig-1 and Vig-3 gene expression in vaccinated and unvaccinated trout after challenge with live IHNV

IFN-1, Mx-1, Mx-3, Vig-1 and Vig-3 gene expression was examined in kidney and spleen of three orally vaccinated fish (10μg dose of pIRF1A-G) 10 days after challenge with IHNV and the results are presented in Figures 6 B and C, respectively. Although no significant differences in the expression of Mx-1, Mx-3, Vig-1 and Vig-3 genes in kidney of vaccinated and unvaccinated fish was evident a significant increase in IFN-1 gene expression was observed 10 days after challenge in spleen of vaccinated fish when compared with unvaccinated fish.

G. Alginate-encapsulated pIRF1A-G vaccine induced the production of anti-IHNV antibodies in the serum of the vaccinated fish

At 15, 30 and 45 days pv. and before challenge with IHNV, blood samples were collected from the caudal vein of orally vaccinated fish (50 or 100μg doses of pIRF1A-G), and from fish injected with 5μg of pIRF1A-G (n = 10). The corresponding serum samples were tested for anti-IHNV antibodies by ELISA. As seen in figure 7, anti-IHNV antibodies were produced in response to the oral or intramuscular administration of pIRF1A-G. Remarkably, no significant differences in antibody production were detected between the three groups of vaccinated fish at the three assessed p. v. time points.

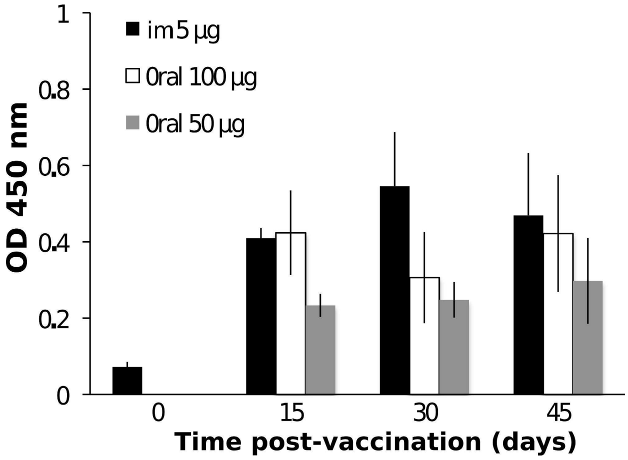

FIG. 7: Alginate-encapsulated pIRF1A-G vaccine induced the production of anti-IHNV antibodies in the serum of the vaccinated fish. At 15, 30 and 45 days pv. and before challenge with IHNV, blood samples were collected from the caudal vein of orally vaccinated fish (50 or 100μg dose of pIRF1A-G), and from fish injected with 5μg of pIRF1A-G (n=10). The corresponding serum samples were tested for anti-IHNV antibodies by ELISAThe bars present the mean antibody levels for 10 fish tested at each time point for each vaccine group. (im: intramuscularly delivered vaccine; cero: mock vaccinated fish).

IV. DISCUSSION

An all-fish DNA vaccine (pIRF1A-G) previously conferred a high level of protection against an IHNV challenge when administered by intramuscular injection (Alonso *et. al.*, 2003). Although effective, injection route is labour intensive and only practiced for high-value species. In the current study, we tested whether the pIRF1A-G vaccine could also be effective when encapsulated in alginate microspheres and delivered orally to rainbow trout. This is the first study that compares intramuscular and oral delivery of the same IHNV DNA vaccine and compares both routes of delivery simultaneously against a challenge with IHNV in rainbow trout.

Following oral immunization with 10μg of pIRF1A-G-loaded alginate microparticles, we were able to detect IHNV G transgene expression by RTqPCR in gills, spleen, kidney, and intestinal tissues of vaccinated fish. The highest IHNV G gene expression levels were recorded in intestinal tissues early after the oral administration of pIRF1A-G (1 and 3 dpv.), suggesting that alginate microparticles effectively protected the DNA vaccine avoiding its degradation in the fish stomach at least to some degree. Form our results, we can also infer that the oral pIRF1A-G vaccine crossed the intestinal epithelium by a still unknown mechanism, was transported into the blood and despite some degradation was able to reach several internal and external organs of the fish. Consequently, IHNV-G transcripts could be detected in spleen, kidney and gills of vaccinated fish at 1, 3, 7 and 15 days after vaccination. When G gene expression in the four examined organs was compared, the lowest expression levels were detected in kidney of vaccinated fish at the four assessed time points. As we previously suggested, the fish head kidney might be acting as a scavenger tissue, clearing the blood from circulating plasmid DNA (Ballesteros *et. al.*, 2012).

As expected, our results demonstrated that the expression of IHNV G transcripts in kidney and spleen of vaccinated trout increased when increasing concentrations of pIRF1A-G loaded alginate microparticles. Although alginate microparticles are very resistant to acidic pH, which impedes degradation of the vaccine in the stomach of the fish (pH 2-4) and favours its release in the forgut and hindgut (pH 7 and 8.3, respectively), our results indicated that the oral route required approximately 20-fold more plasmid DNA than the injection route to achieve significant levels of IHNV G expression in fish internal organs. After vaccine passing through the intestinal epithelium, however, similar IFN-1 and Vig 2 gene expression was detected in kidney and spleen of vaccinated fish at 3, 7 and 15 days pv., regardless of the administration route. Moreover, a low dose of the oral vaccine (10μg) was able to stimulate the expression of IFN-1, TLR-7 and IgM genes in kidney of orally vaccinated fish. In addition, the oral administration of 10μg of pIRF1A-G vaccine resulted in a significant induction of Mx-1, Vig-1, Vig-2 and TL-7 gene expression at 3 d pv. in spleen of vaccinated trout. Remarkably, IFN-1 gene expression was significantly induced by the three tested doses of the oral DNA vaccine (10, 25 and 100μg) in spleen and kidney of vaccinated fish when compared with unvaccinated fish. In contrast, significant Mx-1, Vig-1, Vig-2, TLR-3 and TLR-8 gene expression was only detected when high doses of pIRF1A-G vaccine were orally administered (50 and 100μg).

TLRs are transmembrane proteins that recognise conserved pathogen structures. In fish, several studies have reported that TLRs expression is regulated by viral infections. Our results suggested that TLR-3, TLR-7 and TLR-8 expression was affected by the oral and intramuscular administration of pIRF1A-G DNA vaccine. These TLRs acted, therefore, as important mediators of IHNV DNA vaccines in rainbow trout. Remarkably, the administration of 100μg of the oral vaccine induced higher levels of TLR-3, TLR-7 and TLR-8 gene expression in spleen of the vaccinated animals at day 7 p. v. than did the intramuscular injection of the DNA vaccine. It remains to be elucidated whether trout oral IHNV DNA vaccination with pIRF1A-G-loaded alginate microparticles mimics several other transcriptional responses occurring after infection with IHNV. Previously, we demonstrated that the oral-alginate VP2-vaccination immunizes trout against IPNV in a similar way as IPNV infection does (Ballesteros *et. al.*, 2012).

Although similar levels of IFN-1 and Vig 2 gene expression were detected in kidney and spleen of vaccinated fish regardless of the administration route, several studies have shown that the intramuscular administration of a vaccine stimulates a different immune response from that stimulated by administration of antigens orally (Rombout *et. al.*, 2006; Rombout *et. al.*, 2014). In fish, as in higher vertebrates, there is a very complex mucosa associated lymphoid tissue (MALT) in the gut (GALT), skin (SALT), nasopharynx (NALT) and gills (GIALT), with a specific and specialized immune response for these tissues (Tyagi *et. al.*, 2012; LaPatra *et. al.*, 2015). In the fish intestine, for instance, CD8+ T cells dominate the CD4+ subset and the number of such cells increases from the foregut to the hidgut (Pichetti *et. al.*, 2011). Oral and anal administration in fish activates GALT, while injection generally does not activate this stimulatory route (Salinas *et. al.*, 2011). Although we did not study local GALT immune responses, which might be very important after oral vaccination, CD4 (a classical marker of T helper cells) and CD8 (a marker of cytotoxic lymphocytes) transcripts were measured in response to the oral or intramuscular administration of pIRF1A-G DNA vaccine in internal organs of the vaccinated fish at 15 days pv. Our results showed higher CD4 and CD8 expression levels in kidney of fish vaccinated with 100μg of the oral vaccine than in the im. fish. Intramuscularly infected fish did not show a significant increase in CD8 expression confirming that different vaccine delivery routes stimulate different cellular immune responses. Until recently, teleost fish B cells were thought to express only two classes of immunoglobulins, IgM and IgD, in which IgM was thought to be the only one responding to pathogens both in systemic and mucosal compartments. However, a third teleost immunoglobulin class IgT, similar to zebrafish IgZ, has been recently been shown to behave as the prevalent immunoglobulin in gut mucosal immune responses (Zhang *et. al.*, 2010). Previously, we analysed local production of Igs in one of the fish intestinal segments where mucosal immunity is induced after oral vaccination, the pyloric caeca (Ballesteros *et. al.*, 2012). Increased IgM and IgT expression levels in pyloric ceca compared to kidney were detected after the oral administration of alginate microparticles containing pcDNA-VP2 DNA vaccine (Ballesteros *et. al.*, 2012). In our current study, we measured IgM and IgT production in head kidney (the bone marrow equivalent) and spleen after the oral or intramuscular administration of pIRF1A-G. In teleost fish, the head kidney is considered the primary lymphoid tissue, a key hematopoietic organ, and an important source of Ig-secreting B cells (Salinas *et. al.*, 2011). The spleen, like the head kidney, also contains a large number of B cells and is an organ in which activation and differentiation of B cells occur. Our results showed differential modulation of IgM and IgT after the oral or intramuscular delivery of pIRF1A-G in vaccinated fish at 15 days post-vaccination. While the oral administration of 100μg of pIRF1A-G vaccine resulted in a significant induction of both IgM and IgT gene expression in kidney and spleen of the vaccinated fish, the intramuscular delivery of the vaccine only induced significant levels of IgT expression in spleen of vaccinated fish. No significant production of IgT was detected in kidney of the i.i fish confirming IgT up regulation only when the vaccine was orally administered. In agreement with these results, no significant differences in IgM production were detected in serum samples from intramuscular and oral vaccinated fish at the three assessed pv. time points. However, the efficient induction of a local immune response in the GALT after oral vaccination was shown to induce a more intense immune response than the systemic immunization (Adelman *et. al.*, 2008; Salinas *et. al.*, 2011). Therefore, the detection of antibodies in the gut mucus after the administration of oral DNA vaccines should be a matter of further evaluation. In addition, further research is needed to understand the role of the mucosa-associated IgT in the gut of teleosts. The effect of five doses of the oral pIRF1A-G vaccine on fish CPM was evaluated. Fish injected with 5μg of pIRF1A-G were used as a positive control group. Dose response was evident for the oral pIRF1A-G treatment groups. Statistically significant differences in mortalities between the fish orally vaccinated with 10, 20, 25 and 50μg of the oral DNA vaccine and the empty-plasmid vaccinated or unvaccinated fish were observed. The oral administration of 100μg of pIRF1A-G encapsulated in alginate microparticles to rainbow trout protected against the infection, reaching a RPS value of 56, which is less than the RPS obtained when 5μg of pIRF1A-G vaccine were im. (RPS=70). However, In in a previous study, i. m. of 5μg of pIRF1A-G resulted in protection levels up to 55 RPS (Alonso *et. al.*, 2003). RPS differences between both studies might be explained by difference in mortalities in control treatment groups. Other authors previously assayed oral DNA vaccines against IHNV encapsulated by the polymer poly-(D,L-lactic-coglycolic acid) (PLGA) and added to feed (Adomako *et. al.*, 2012). The induced protection was much lower than that achieved with the intramuscularly injected vaccine and was also lower than that described here for the alginate-encapsulated pIRF1A-G vaccine. However, both alginate and PLGA carrier systems for encapsulation seem to provide accurate plasmid coating, as persistence of transgene expression was observed in several tissues after vaccination.

In summary, our results demonstrated that alginate microspheres protected the pIRF1A-G vaccine which was expressed in several organs of the vaccinated trout. A low dose of the oral vaccine (10μg) was able to stimulate the expression of IFN-1, TLR-7 and IgM gene in kidney of orally vaccinated fish. In addition, the oral administration of 10μg of pIRF1A-G vaccine resulted in a significant induction of Mx-1, Vig-1, Vig-2 and TL-7 gene expression at 3 d pv. in spleen of vaccinated trout. In contrast, significant Mx-1, Vig-1, Vig-2, TLR-3 and TLR-8 gene expression was only detected when high doses of pIRF1A-G vaccine were orally administered (50 and 100μg). These high doses of the oral DNA vaccine induced low levels of anti-IHNV antibodies and protected 45 and 56% of the vaccinated trout, respectively. Therefore, there is still need for improvement of this oral

IHNV vaccine. An approach to reduce the amount of pIRF1A-G required for oral vaccination and to increase its immunogenicity of the vaccine might be the administration of encapsulated mucosal adjuvants such as traditional aluminium salts, polysaccharides (e. g. zymosan, glucans, chitosan), and TLR agonists (Holvold *et. al.*, 2014). Moreover, oral administration of plasmids encoding cytokine adjuvants (IL-2, IFN-, IL-12, GM-CSF and IL-15) may be another approach to increase the immunogenicity of the oral pIRF1A-G vaccine (Caipang *et. al.*, 2009). As CD8 + cells dominate the CD4+ subset in the intestine, oral DNA vaccines should be developed to specifically target these effector immune cells at the mucosal surfaces as a way to increase their efficacy. Induction of log-term memory by specific specific stimulation of mucosa-associated IgT might also be considered. Perhaps such improvements could also increase the efficacy of the oral pIRF1A-G vaccine in terms of survival (> 56 RPS). In other hand, it remains to be determined whether the addition of the oral pIRF1A-G vaccine to food pellets could serve as an efficient method of inducing protective immune responses against IHNV infection. Using this approach, the plasmid could be easily delivered in multiple successive events, avoiding fish stress and likely increasing vaccine effectiveness. Previously, we demonstrated that oral vaccine delivery through feeding is a promising delivery method, at least for IPNV vaccines (Ballesteros *et. al.*, 2012).

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5. Determinar masivamente los perfiles de transcripción de trucha arco iris en respuesta a la infección con el virus de la Necrosis Hematopoyética Infecciosa.

La vacuna frente a IHNV por vía oral proporcionó resultados positivos con dosis altas aunque comparativamente peores que los obtenidos con la misma vacuna por vía intramuscular, por lo cual, será necesario optimizar este sistema para obtener mayor eficiencia y protección con esta vacuna y vía de administración. Para lograrlo un primer paso es conocer mejor la infección del virus, las alteraciones que provoca en el hospedador y su repercusión en el sistema inmune. En esta línea se ha utilizado también el modelo trucha arco-iris, que pese a su dificultad por ser una especie aún no bien conocida a nivel genómico, es sin embargo uno de los hospedadores naturales del virus IHN. El experimento fue llevado a cabo en dos momentos de la infección viral, un tiempo de infección inicial o asintomática (3 dpi.), y un tiempo de infección sintomático o clínico (7dpi.), utilizando la técnica de secuenciación masiva. Los resultados forman parte de un primer trabajo que está preparándose para enviar a publicar y cuya primera descripción se adjunta como resultados en este objetivo.

Introducción a la metodología.

La secuenciación masiva de RNA (RNA-seq) es un método eficiente para el descubrimiento y anotación tanto de genes de codificación como para no codificantes. La técnica de RNA-seq no requiere de clonación ni genotecas, permitiendo la secuenciación directa de los fragmentos de cDNA. Esta técnica ha sido ampliamente utilizada para el estudio de las alteraciones de las vías celulares durante infecciones y los cambios en la expresión de genes (Nagalakshmi, Waern, and Snyder 2010). La técnica de RNA-seq ha demostrado ser altamente precisa y reproducible para cuantificar los niveles de expresión, y por último, al no requerir pasos de amplificación, se requiere menos cantidad o muestra de RNA (Shi and He 2014). A diferencia de los enfoques basados en hibridación, la técnica de RNA-seq no se limita a la detección de transcritos que corresponden a la secuencia genómica existente, por lo cual es una técnica que puede ser utilizada en cualquier especie, aunque no tenga determinada su secuencia genómica.

Por otra parte, la técnica de RNA-seq puede revelar la ubicación precisa de los límites de transcripción, a una resolución de una sola base. Además, al tener lecturas cortas de tan solo 30 pb puede ser muy útil en información acerca de cómo se conectan dos exones ya que las lecturas cortas deben revelar la conectividad entre múltiples exones. Estos factores hacen que el RNA-seq sea muy útil para el estudio de transcriptomas complejos. Además, también puede revelar variaciones de secuencias como SNPs en las regiones transcritas (Salem et al. 2012). En resumen, la técnica de RNA-seq es el primer método de secuenciación que permite que todo el transcriptoma sea objeto de reconocimiento en un muy alto rendimiento y forma cuantitativa (Veytsman et al. 2014). Para analizar los datos de secuenciación masiva en primer lugar debemos tomar como base una referencia (genoma o transcriptoma) para agrupar los datos en contigs y así revelar la estructura de la transcripción. Los programas disponibles para la cartografía o alineación de las lecturas son Bowtie, BWA, SOAP, entre otros (Eveillard et al. 2001; Langmead and Salzberg 2012; Li and Durbin 2009). Sin embargo, existen lecturas de transcriptomas cortas como los extremos poli A o cruces de exones que no pueden ser analizados de la misma manera; por lo cual estos transcriptomas complejos difíciles de mapear por la extensa cantidad de genes con *splicing* alternativo y trans-empalme, son compilados en bibliotecas que contienen todas las secuencias de unión, mapas conocidos y lecturas previstas a varias librerías. Un reto consiste en el desarrollo de métodos computacionales simples para identificar nuevos eventos de alineaciones producidas entre dos secuencias distantes o entre los exones de dos genes diferentes (Trapnell et al. 2012). Para grandes transcriptomas, la alineación también se complica por el hecho de que una porción significativa de la secuencia puede alinearse con varias ubicaciones en el genoma, por lo cual se han utilizado métodos más consistentes para la alineación como aquellos que utilizan algoritmos de cadenas de Markov entre otros, los cuales bareman la posición y alineación de las lecturas según su secuencia vecina (Sperschneider et al. 2013).

Amplia complejidad de transcripción

La técnica de RNA-seq puede ser utilizada para examinar cuantitativamente la diversidad de secuencias, ya que puede capturar la dinámica transcriptómica a través de diferentes muestras o condiciones sin normalizaciones sofisticadas de los datos, por lo cual proporciona una "medida digital" de la diferencia de expresión de los genes entre diferentes muestras.

Existen dos métodos para el procesamiento de las lecturas del transcriptoma:

De novo: Este método no utiliza un genoma de referencia para reconstruir las secuencias del transcriptoma, por lo cual se recomiendan lecturas amplias de al menos 100 pb para no encontrar grandes coincidencias entre las lecturas y evitar muchas regiones con secuencias repetitivas. Los software disponibles para esta clase de procesamientos de datos son Trinity, Velvet y Oasis (Ashrafi et al. 2012; Huth and Place 2013; Ioannidis et al. 2014).

Con genoma o transcriptoma de referencia: En este caso, es más fácil la alineación de los millones de lecturas con un genoma o transcriptoma de referencia, aunque se debe tener precaución al alinear un transcriptoma respecto a un genoma, sobre todo cuando los genes tienen regiones intrónicas. Existen varios paquetes de software para la alineación de lecturas y algoritmos especializados para la alineación del transcriptoma, por ejemplo, Bowtie para RNA-seq con lecturas cortas, TopHat para alineación de sitios de empalme en un genoma de referencia, Cufflinks que utiliza un modelo matemático riguroso para identificar el conjunto completo de los transcritos regulados alternativamente en cada locus y para asignar la cobertura para cada transcripción (Anders and Huber 2010; Ozer, Parvin, and Huang 2012; Trapnell et al. 2012). Para el desarrollo de esta tesis, fue necesario establecer la metodología del análisis de datos en trucha arco iris infectada con IHNV, debido a que hasta la fecha se ha estudiado a nivel transcripcional la expresión inmune ocasionada por diferentes estímulos utilizando RTqPCR y microarrays; pero hasta el momento no se han descrito trabajos en donde se evalúe masivamente a nivel transcripcional la expresión génica ocasionada por el virus IHN en dos momentos diferentes de la infección, a los 3 días en donde no se detectan signos clínicos y a los 7 días en donde se evidencia toda la sintomatología. En este trabajo, se describe la expresión génica en estos dos momentos utilizando la técnica de RNA-seq.

Diseño experimental:

Figura 1: Diseño experimental

Alevines de trucha arco iris de aproximadamente 2 g, 6 cm de longitud y 4 meses de edad

- Grupo de peces (n=20) infectados con IHNV por inmersión durante 2 horas. A los 3 días post-infección se sacrificaron 6 peces y se les extrajo el RNA total de tejido renal. El grupo se denomina “infectados 3 días o asintomáticos)
- Grupo de peces (n=20) infectados con IHNV por inmersión durante 2 horas (n= 20). A los 7 días post-infección se sacrificaron 6 peces y se les extrajo el RNA total de tejido renal. El grupo se denomina “Infectados 7 días o sintomáticos”.
- Grupo de peces sin tratamiento n=20 “Control”. Se toman muestras en paralelo a las anteriores.

Tiempos de muestreos: 3 y 7 días post-infección.

Muestra: riñón anterior.

Métodos experimentales: Extracción de RNA total utilizando el kit Quiagen, electroforesis de capilar y secuenciación masiva RNA-seq.

Protocolo:

Una vez extraído el RNA del riñón anterior se realizó el pool de 6 RNAs de individuos diferentes, y de éste se analizó la integridad del RNA utilizando el software y equipo Experion ™ (Biorad), que determina el 'Número de la integridad del RNA (RQI o RIN)', utilizando un algoritmo para la asignación de valores de integridad a las mediciones de RNA eucariota, que es evaluada utilizando la relación del rRNA del 28S respecto al 18S, además de una combinación de mediciones electroforéticas de RNA. Para el experimento de RNA-seq se requieren valores de RQI altos, mayores a 9 como se observa en una de las muestras obtenidas, Figura 2.

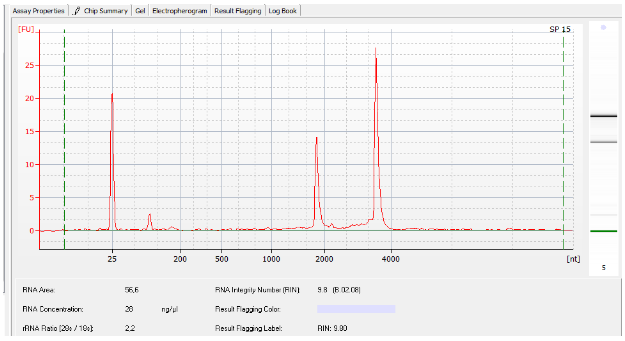

Figura 2: Gráfica generada por el equipo de Agilent Technology acerca de la calidad del RNA de una de las muestras para secuenciación masiva.

Una vez comprobado que las muestras tengan la integridad adecuada, son aceptadas para la secuenciación y los datos obtenidos deben contar un pre-procesamiento.

Pre-procesamiento de los datos: El Parque Científico de Madrid, donde se realizó la secuenciación masiva, nos brinda los datos ya con un pre-procesamiento, por ello en nuestro caso omitimos describir con detalle este paso; no obstante, en esquema se requieren varios pasos que, pueden realizarse a través de varias herramientas informáticas como "fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html)" y consta de tres etapas fundamentales: En la primera se eliminan los 10 primeros nucleótidos de todas las secuencias, eliminando así una de las fuentes de desviación y que contiene elementos sobre-representados; en la segunda etapa se eliminan las secuencias con una puntuación de calidad ≤20 en el 80% de sus posiciones, y en la tercera etapa se eliminan las secuencias con más de 5 posiciones indeterminadas, los adaptadores utilizados en la carrera y las secuencias o contaminantes menores a 50 bases. Una vez finalizado este proceso, las secuencias son separadas en ficheros independientes y correspondientes a cada muestra. Una vez secuenciada la muestra y pre-procesados los datos, nosotros analizamos la calidad de las lecturas o "reads" producidas; para ello existen varios programas informáticos (software) que proporcionan herramientas para el control de la calidad de datos de secuenciación. En nuestro caso, utilizamos el programa informático FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) para detectar y (si es necesario) eliminar los errores y zonas de baja calidad en las secuencias, conservando las secuencias de mayor calidad y manteniendo la máxima cantidad posible de información del experimento.

Esta herramienta nos brinda información acerca las características de nuestros resultados, como la calidad de la secuencia por posición, en donde se pueden observar en el eje Y del gráfico los valores de calidad en un intervalo de 0 a 40, divididas en zonas de muy buena calidad (verde, de 28 a 40), calidad media (naranja) o baja calidad (rojo) Figura 3A. Además de la distribución de la longitud de las lecturas como se puede observar en la figura 3B, en la cual en el eje X se representan las longitudes de las secuencias en pares de bases y en el eje Y se indica el número de secuencias con una longitud determinada. Cuanto menor sea la desviación en la longitud de las secuencias, el análisis de éstas será más sencillo.

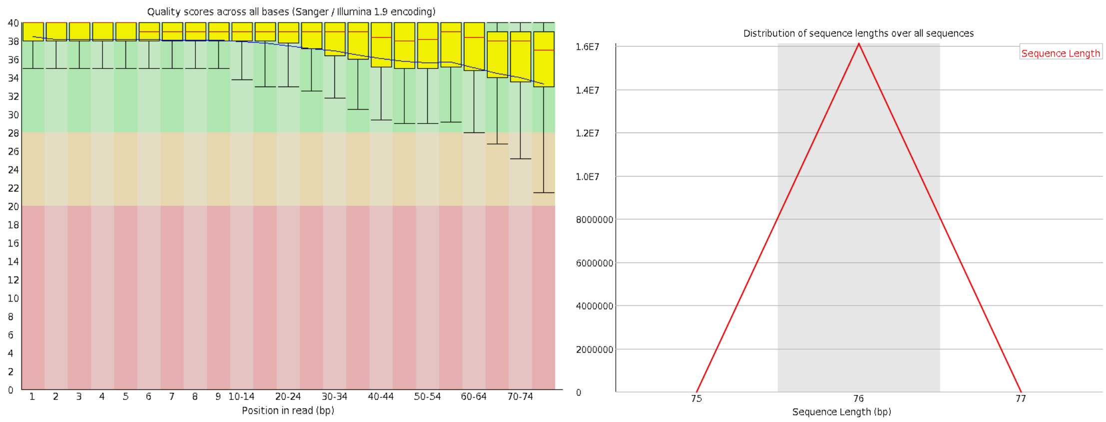

Figura 3: Gráficas generadas por el programa fastqc para los resultados de secuenciación masiva de una de las muestras. (A). Calidad de las secuencias por base. (B). Distribución del tamaño de las lecturas secuenciadas.

Existen otros parámetros con los que igualmente se evalúa la calidad de las lecturas obtenidas; entre ellos se encuentran: la distribución del contenido de las secuencias por base, la distribución del contenido de GC por secuencia y por base, la distribución del contenido de N por base y finalmente, la distribución de secuencias duplicadas (Figuras no mostradas).

Es de aclarar que nuestras secuencias cumplieron los requisitos de calidad necesarios, por lo cual continuamos con su análisis, siguiendo el siguiente esquema:

5.1.1. Selección de las herramientas informáticas para la alineación y,

5.1.2. Selección de referencias para el alineamiento.

5.1. Obtención de los parámetros adecuados para el análisis de los datos de RNA-seq en trucha arcoíris

El transcriptoma fue seleccionado como referencia para establecer los parámetros adecuados para el análisis. Esta referencia (transcriptoma) fue utilizada de dos formas diferentes, la primera correspondió al transcriptoma o CDs tal cual, y la segunda forma correspondió al transcriptoma dividido en dos subgrupos, genes ohnologos (genes duplicados) y genes no-ohnologos (genes únicos). Los subgrupos del transcriptoma fueron establecidos con el fin de obtener una expresión génica más exacta y precisa de los genes expresados o subexpresados con los distintos tratamientos, disminuyendo así el error en la estimación de la expresión causado por la alineación de nuestras lecturas (reads) en dos o más lugares de la secuencia utilizada como referencia; ello es debido a que los salmónidos descienden de un antepasado autotetraploide, causado por la duplicación del genoma completo (Allendorf and Thorgaard 1984). Esta cuarta ronda de duplicación del genoma completo (4R) es relativamente reciente en términos de evolución, pues ocurrió hace 88 a 103 millones años (MYA) (Alexandrou et al. 2013). Sin embargo, los genomas de los salmónidos están regresando a un estado diploide estable a través de reordenamientos cromosómicos y divergencias de cromosomas homólogos; parte de la evidencia de que la dipliodía aún no está completada se observa en la herencia tetrasómica actual en los machos y la extensa reorganización entre los cromosomas (Allendorf and Thorgaard 1984). Estos cromosomas duplicados se han caracterizado por la pérdida de aproximadamente la mitad de las regiones codificantes de proteínas a través de procesos de pseudo-genizacion. Sin embargo, la mayoría de la retención de los genes duplicados se han caracterizado como miRNA (Berthelot et al. 2014). Por lo tanto, hemos utilizado las secuencias Ohnologos y No-Ohnologos como referencia para el alineamiento, con el fin de representar y obtener la mayor y máxima expresión de la transcripción en los genes únicos y duplicados, como ya se mencionado.

Por otra parte, se han comparado varios algoritmos para la optimización de la alineación de las lecturas respecto a las secuencias utilizadas como referencia (transcriptoma, genes ohnologos y no-ohnologos), tales como Bowtie *(http://bowtie-bio.sourceforge.net/index.shtml)* versión 1.1.0 - 7/19/2014 (Langmead et al. 2009), Bowtie2 *(http://bowtie-bio.sourceforge.net/bowtie2/index.shtml)* Versión 2.2.3 - May 30, 2014 (Langmead and Salzberg 2012), y BWA *BWA (http://bio-bwa.sourceforge.net/)* (Li and Durbin 2009), los cuales utilizan el método de Burrows-Wheeler Aligner.

Las principales diferencias entre estos programas consisten en la exigencia y restricción de las alineaciones, ya que el algoritmo para Bowtie exige una alineación en los bordes o extremos de las secuencias analizadas, mientras que para Bowtie2 y BWA, el algoritmo permite y reporta las alineaciones entre las secuencias, así éstas no alineen en los extremos. Los datos de RNA-seq fueron analizados utilizando estos tres programas con el fin de obtener el mejor rendimiento del algoritmo para lograr la mayor alineación y el menor error. En nuestro caso, con Bowtie2 obtuvimos un error del 11% respecto al 16 y 20% con BWA y Bowtie respectivamente. Por otra parte, también se han definido los mejores parámetros para cada uno de los pasos, como en el ensamblaje, cuantificación, normalización y finalmente, en la determinación del valor relativo de expresión de los genes, tanto para Bowtie2 como para Cufflinks *(http://cufflinks.cbcb.umd.edu/downloads/):* versión v3.0.2 (Trapnell et al. 2012).

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Bowtie2 contiene un algoritmo más rápido respecto a las otras herramientas, debido a que procesa las secuencias con un uso eficiente de memoria, ya que es una herramienta que opera bien con lecturas de tamaño entre 50 y 100 (aquí los reads son de 75 bp). Mientras que Bowtie procesa mejor lecturas más pequeñas de 50 bp, siendo más rápido y sensible con este tipo de datos. Y finalmente, BWA, resulto ser una herramienta menos sensible por lo que se pierden más lecturas en la alineación y además, tarda más tiempo en realizar el proceso de los datos.

A continuación se muestran los resultados de alineación de las lecturas obtenidas con los diferentes métodos de alineación (Bowtie, Bowtie2 y BWA) para las siguientes referencias:

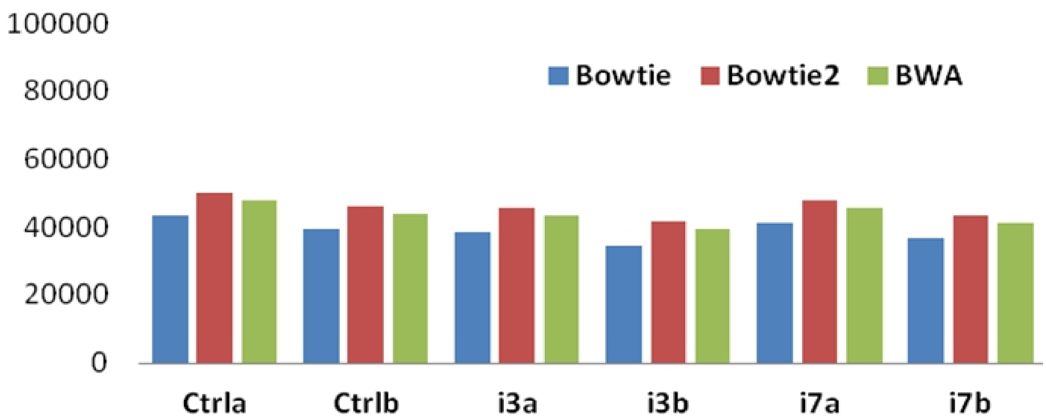
CDs, bajo el parámetro de alineación k=1, con el fin de obligar al algoritmo a alinear una única vez la lectura a la referencia, y bajo el parámetro de alineación k=6, con el fin de permitir al algoritmo alinear más de una vez y máximo 6 veces la lectura respecto a la referencia.

No_ohnologos, bajo el parámetro de única alineación por lectura k=1 y, Ohnologos, bajo el parámetro de múltiples alineación por lectura k=6,

5.1.1. Diferentes Herramientas de Alineación

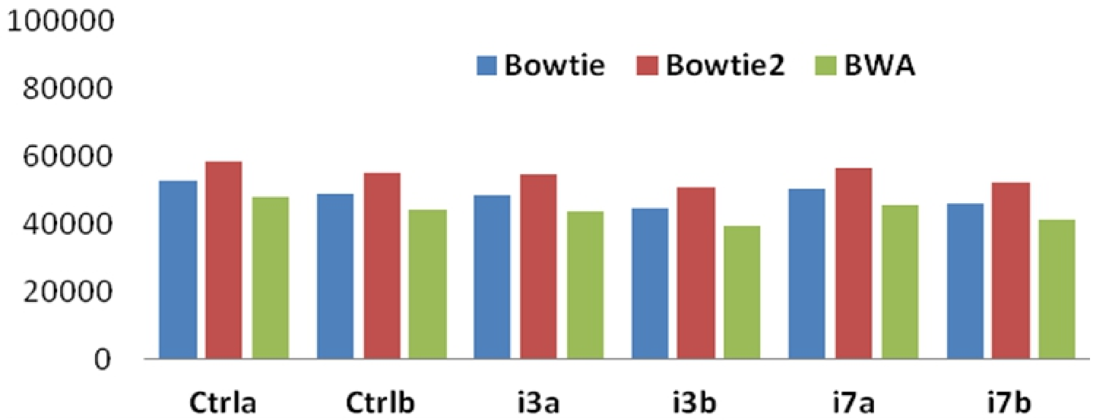
5.1.1.1. CDs como referencia de alineación, bajo el parámetro de una única alineación (k=1) y múltiple alineación k=6:

En las figuras 4 y 5 se presentan los resultados obtenidos utilizando alineación única o múltiple. En todas las muestras evaluadas el mejor resultado se obtuvo con la alineación realizada con Bowtie2 frente a los resultados con Bowtie y BWA. Se utilizó como referencia el transcriptoma "CD" con parámetros de alineación de una única alineación (k=1) además de alineaciones múltiples de las lecturas respecto a la secuencia de referencia (k=6). Los resultados señalan que con el método de Bowtie se consiguió aproximadamente la misma cantidad de lecturas alineadas en comparación con BWA (alrededor un 90% y 115%, respectivamente), mientras que utilizando el método de Bowtie2 se obtuvo una leve mejora de aproximadamente 105% y 120% de lecturas alineadas, respectivamente. Ver tablas 1 y 2.



Muestra	Bowtie vs. BWA (%)	Bowtie2 vs. BWA (%)
Ctrla	91	105
Ctrlb	89	105
i3a	89	106
i3b	88	106
i7a	90	105
i7b	89	106
cuffmerge	182	207
cufflinks	91	103

Figura 4 y Tabla 1: Secuencia CDs con diferentes herramientas de alineación, con el parámetro k=1. Cantidad de lecturas alineadas en cada una de las muestras de los peces infectados.

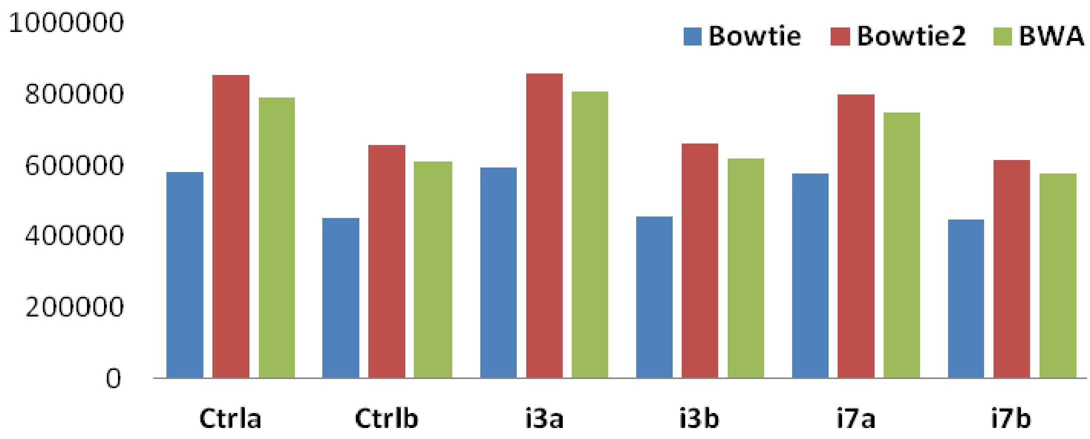


Muestra	Bowtie vs. BWA (%)	Bowtie2 vs. BWA (%)
Ctrla	110	122
Ctrlb	112	125
i3a	111	125
i3b	113	129
i7a	111	124
i7b	112	128
cuffmerge	108	234
cufflinks	108	117

Figura 5 y Tabla 2: Secuencia CDs con diferentes herramientas de alineación, con el parámetro k=6. Cantidad de lecturas alineadas en cada una de las muestras de los peces infectados.

5.1.1.2. Secuencia de No-Ohnologos como referencia de alineación, bajo el parámetro de única alineación por lectura, k=1:

El mejor resultado en todas las muestras evaluadas corresponde a la alineación con Bowtie2. Se observa en la figura 6 y tabla 3 que al determinar el porcentaje de lecturas alineadas cuando se utilizan los No-Ohnologos como referencia con un parámetro de alineación de una única lectura por mapeo, de los tres métodos utilizados (Bowtie, Bowtie2 respecto a BWA). El método de Bowtie consiguió una menor cantidad de lecturas alineadas en comparación con BWA (alrededor de un 75%), mientras que utilizando el método de Bowtie2 se obtuvo un resultado algo mejor de aproximadamente el 105% de lecturas alineadas.

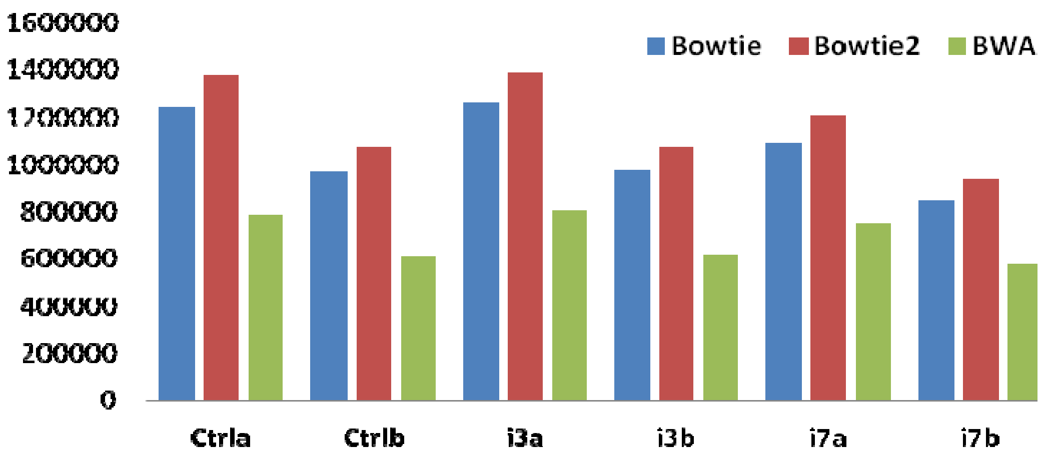


Muestra	Bowtie vs. BWA (%)	Bowtie2 vs. BWA (%)
Ctrla	74	108
Ctrlb	74	107
i3a	73	106
i3b	74	107
i7a	77	107
i7b	78	107

Figura 6 y Tabla 3: No-ohnologos o genes únicos con diferentes herramientas de alineación (Bowtie, Bowtie2 y BWA) con el parámetro k=1. Cantidad de lecturas alineadas en cada una de las muestras de los peces infectados.

5.1.1.3. Secuencua de Ohnologos como referencia de alineación, bajo el parámetro de múltiples alineaciones por lectura, k=6:

Según la figura 7 y tabla 4, el mejor resultado en todas las muestras evaluadas corresponde nuevamente a la alineación con Bowtie2, ya que al realizar el porcentaje de lecturas alineadas cuando se utilizan los Ohnologos como referencia con un parámetro de alineación de múltiples lecturas por mapeo, de los tres métodos utilizados (Bowtie, Bowtie2 respecto a BWA) se comprobó que con el método de Bowtie se consiguió aproximadamente la misma cantidad de lecturas alineadas en comparación con BWA de alrededor de un 156%, mientras que utilizando el método de Bowtie2 se obtuvo un ligero aumento de resultados de aproximadamente el 170% de lecturas alineadas.



Muestra	Bowtie vs. BWA (%)	Bowtie2 vs. BWA (%)
Ctrla	157	174
Ctrlb	158	176
i3a	156	172
i3b	157	173
i7a	146	161
i7b	147	163

Figura 7 y Tabla 4: Ohnologos o genes únicos con diferentes herramientas de alineación, con el parámetro k=6. Cantidad de lecturas alineadas en cada una de las muestras de los peces infectados (en porcentaje).

Como resultado final, se determina Bowtie2 como la mejor herramienta para la alineación de nuestras lecturas o reads tanto para el CDs como para los No-ohnologos y Ohnologos cuando son utilizados como referencia, por esta razón, se continua con el análisis de los datos utilizando Bowtie2.

5.1.2. Diferentes Referencias para la Alineación

En el caso de la segunda condición, se utilizan tres referencias diferentes para la alineación de los datos como el genoma, el CDs o transcriptoma sin los genes de regulación o promotores, y finalmente el CDs pero dividido en los dos subgrupos anteriormente descritos (ohnologos y No-Ohnologos).

5.1.2.1. Genoma como referencia de alineamiento

La secuencia del genoma fue obtenida según Berthelot (2014) (Berthelot et al. 2014), y descargada de la página web (http://www.genoscope.cns.fr/trout-ggb/data/Oncorhynchus_mykiss_cds.fa.gz) consultada el 05-2014.

Es de recordar que para el procesamiento de estos datos se utilizan los programas informáticos TopHat y Cufflinks. TopHat (*http://tophat.cbcb.umd.edu/downloads/*) tiene dependencias de Bowtie2 (versión 2.0.0) (Trapnell et al. 2012). Por otra parte, la herramienta Samtools fue utilizada en todo momento con el fin de modificar el formato de los archivos obtenidos (*http://samtools.sourceforge.net/*) (Li et al. 2009). Los resultados obtenidos al utilizar el genoma como referencia pueden resumirse en la tabla 5:

Tabla 5: Tabla de alineación de las lecturas respecto al genoma como referencia.

Muestras	Nº lecturas únicas mapeadas	% lecturas únicas mapeadas	% lecturas multi mapeadas	% total lecturas mapeadas
Control (ctr)	9’877.017	52,81%	26,85%	79,66%
	7’699.081	52,83%	26,55%	79,38%
Infectados 3 días (i3)	10’107.793	49,20%	29,42%	78,62%
	7’818.957	49,59%	29,07%	78,66%
Infectados 7 días (i7)	9’767.038	56,49%	24,08%	80,57%
	7’601.547	56,54%	23,98%	80,52%

En la tabla 5, *se* observa que en promedio ha mapeado el ~52% de las lecturas únicas y el ~26% de lecturas han multimapeado en diferentes zonas del genoma.

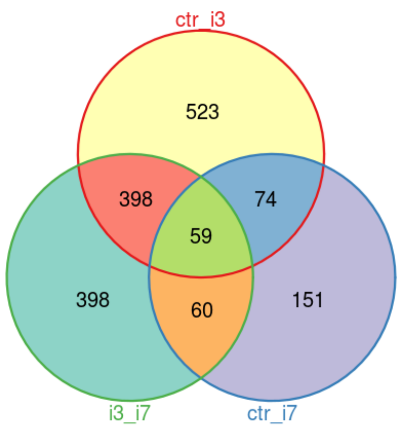


Figura 8: Diagrama de Venn con los transcritos estadísticamente significativos entre los diferentes grupos (Infectados 3 dpi/Control; Infectados 7 dpi/Control) al utilizar el genoma como referencia de alineamiento.

A pesar de obtener la mayoría de las secuencias alineadas, finalmente no se obtiene mayores niveles de expresión estadísticamente diferentes entre los transcritos de las diferentes condiciones (Figura 8).

Se ha utilizado el CDs como única referencia de alineación, y el CDs dividido en dos subgrupos (ohnologos y no-ohnologos) como referencias de alineación. La distribución de estos ficheros se menciona a continuación:

- CDS: contiene 46.585 contigs (todos los genes)
- Non-ohnologos: contiene 32.468 contigs (genes únicos) y Ohnologos: contiene 14.117 contigs (genes duplicados)

5.1.2.2. CDs como referencia de alineamiento

En peces infectados con IHNV, la expresión de transcritos estadísticamente diferenciados (DETs) se dividieron en los siguientes grupos: 3292 genes en el grupo de peces infectados 3 días versus peces controles (i3/ctr); 2736 genes en el grupo de infectados 7 días versus controles (i7/ctr); 3401 genes en el grupo de infectados 3 días versus infectados 7 días (i3/i7); de los cuales, 798 genes son similares en los grupos (i7/ctr vs. i3/i7), 1180 genes similares entre los grupos (i3/ctr vs. i3/i7), 646 genes comunes entre los grupos (i3/ctr vs. i7/ctr) y finalmente 789 genes comúnmente expresados en todos los grupos evaluados.

Partiendo de estos genes diferencialmente expresados se procede a la búsqueda de pathways o rutas, utilizando dos métodos: a través de los términos GO y a través de la base de datos KEGG.

Es de aclarar, que previo a la búsqueda de pathways, todos los genes diferencialmente expresados en cada uno de los grupos mencionados anteriormente deben ser ¨tratados¨ ya que, no corresponden a datos de una especie comúnmente utilizada o de referencia en estas bases de datos. El término de datos “tratados” indica una serie de pasos que incluye Blastx y la búsqueda de los términos GO a través de la base de datos como swissprot previo a la búsqueda de pathways en las bases de datos de KEGG y Blast2GO.

En la tabla 6, se puede observar el resumen de las lecturas totales mapeadas con el CDs de *Oncorhynchus mykiss*. Se puede observar que en promedio han mapeado el ~48% de las lecturas únicas y el ~25% de lecturas han multimapeado en diferentes zonas.

Tabla 6: Tabla de alineación de las lecturas respecto a la referencia de CDs.

Muestras	Nº lecturas únicas mapeadas k=1	% lecturas únicas mapeadas k=1	Nº lecturas multi mapeadas k=6	% total lecturas mapeadas
Control (ctr)	9'069.225	50,36%	27,53%	77,89%
	7'123.725	50,89%	27,80%	78,69%
Infectados 3 días (i3)	9'071.948	49,54%	25,94%	75,48%
	7'050.160	50,07%	26,21%	76,28%
Infectados 7 días (i7)	9'202.612	47,49%	23,98%	71,47%
	7'176.153	40,08%	24,26%	64,34%

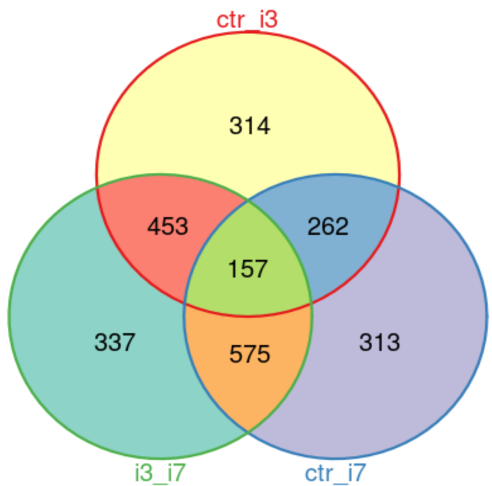


Figura 9: Diagrama de Venn con los transcritos estadísticamente significativos entre los diferentes grupos (Control/Infectados 3 días; Control/Infectados 7días) al utilizar el CDs como referencia de alineamiento.

Al utilizar como referencia de alineamiento el CDs (Tabla 6) y a pesar de obtener menor cantidad de secuencias alineadas a lo obtenido con el genoma (Tabla 5), se obtiene mayor cantidad de transcritos con niveles de expresión estadísticamente diferentes entre las diferentes condiciones, ver figura 9.

5.1.2.3. No-ohnologos y Ohnologos como referencia de alineamiento

La tabla 7 y figura 10 corresponde al resumen de las lecturas totales mapeadas con las secuencias de No-Ohnologos y Ohnologos de *Oncorhynchus mykiss*. Se puede observar que han mapeado aproximadamente el 40% de las lecturas únicas y aproximadamente el 13% de lecturas han multimapeado en diferentes zonas.

Tabla 7: Alineación de las lecturas respecto a la referencia de No-ohnologos y Ohnologos.

Muestras	Nº total lecturas	% lecturas únicas mapeadas (No-ohnol)	% lecturas multi mapeadas (Ohnol)	% total lecturas mapeadas
Control (ctr)	9’069.225	39,87%	12,85%	52,72%
	7’123.725	40,26%	12,98%	43,86%
Infectados 3 días (i3)	9’071.948	40,05%	12,37%	52,42%
	7’050.160	40,47%	12,49%	52,96%
Infectados 7 días 7 (i7)	9’202.612	36,43%	13,37%	49,8%
	7’176.153	36,86%	13,52%	50,38%

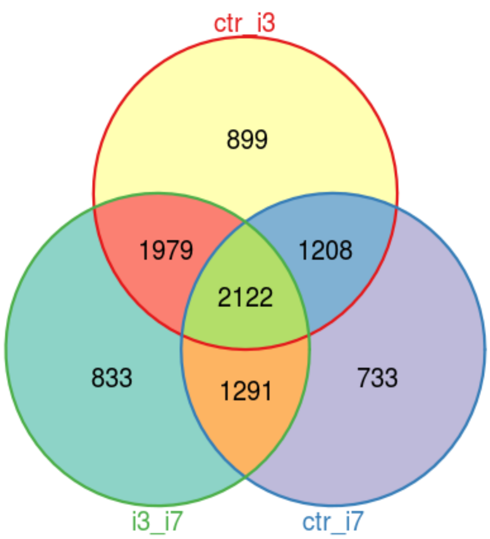


Figura 10: Diagrama de Venn con los transcritos estadísticamente significativos entre los diferentes grupos (Infectados 3 dpi/Control; Infectados 7dpi/Control) al utilizar los No-Ohnologos y Ohnologos como referencia de alineamiento.

Utilizando las secuencias No-Ohnologos y Ohnologos como referencia de alineamiento (Tabla 10), obtuvimos la mayor cantidad de transcritos estadísticamente significativos entre las diferentes condiciones (día 3 y 7 días de infección), como se resume en la figura 11:

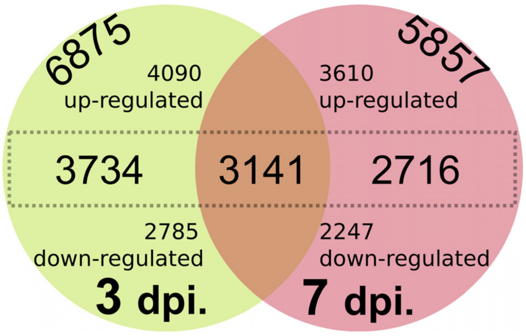


Figura 11: Diagrama de Venn de los transcritos en el grupo de infectados a los 3 y 7 días.

Debido al incremento de transcritos obtenidos tras analizar los datos con los No-ohnologos y Ohnologos como referencia (Figura 11), utilizamos únicamente esta referencia para el resto del análisis.

5.2. Evaluación del perfil de respuesta de genes obtenido mediante secuenciación masiva (RNA-seq) en riñón de trucha arco iris a los 3 y 7 días post-infección con IHNV.

Los resultados finales consisten en listas de genes seleccionados ordenados de mayor a menor según su valor de expresión diferencial. Para ello, se han seleccionado los genes con un valor $p<0.05$ ajustándolos mediante el método descrito por Benjamini & Hochberg (1995) (Benjamini and Hochberg 1995) y así obtener un control estricto sobre el False Discovery Rate (FDR). Estos cálculos para determinar el nivel de transcripción de los transcritos e isoformas y su normalización mediante FPKM fueron realizados mediante el software Cufflinks 2.0.2 (Trapnell et al. 2012), y para el cálculo de expresión diferencial se ha usado la herramienta Cuffdiff propia de Cufflinks (Trapnell et al. 2013). Una vez obtenidos los genes estadísticamente significativos (niveles de expresión respecto al control), se realizó un filtrado para obtener los genes con un ln (fold change) >2 y <-2 veces con un valor $p<0.05$. Una vez obtenida esta información, el siguiente paso consiste en analizar los genes obtenidos a nivel biológico, para ello, se pueden utilizar diferentes metodologías, por ejemplo determinar los términos de gene Ontology (GO) de los genes obtenidos, con el fin de ayudar a responder preguntas como si los genes presentes en la lista tienen funciones similares o si participan en los mismos procesos, y por supuesto también determinar cuáles son y cómo estos procesos están relacionados. La base de datos de GO contiene anotaciones genéricas (independientes de especie) que describen en a.) Funciones Moleculares, b.) Procesos Biológicos o, c.) Componentes Celulares asociados con cada gen.

El análisis de estos datos se organiza de forma jerárquica relacionando todos los términos en niveles sucesivos. Este proceso fue llevado a cabo a través del software “Blast2Go” (https://www.blast2go.com/) (Conesa et al. 2005). Por otra parte, se realizaron análisis de enriquecimiento de genes basados en vías biológicas o pathways conocidos y depositados en bases de datos como la Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/kegg/) (Kanehisa 2000), utilizando la aplicación denominada KASS (KEGG Automatic Annotation Server) para proveer la anotación funcional de los genes a través de comparaciones de Blast y la base de datos de genes de KEGG. El resultado contiene el término KO (KEGG Orthology) asignado por la herramienta KEGG pathways (http://www.genome.jp/tools/kaas/) (Moriya et al. 2007). El siguiente esquema (Figura 12) resume el procedimiento final del análisis llevado a cabo con los datos de RNA-seq.

Resumen:

Se identificaron genes claves en la infección inicial a los 3 días post-infección y a los 7 días post-infección con IHNV, además de algunas rutas “pathways” involucradas en el sistema inmune de trucha arcoíris. A los 3 dpi., se identificaron 6.875 transcritos estadísticamente significativos (DETs) y 5.857 a los 7 días post infección.; de ellos, 3141 DETs fueron comunes entre ambos grupos. A los 3 días post infección, además de encontrar mayor cantidad de DETs respecto a los 7 días., también se evidenciaron pathways más enriquecidos con genes.

Por último, nuestros datos muestran una respuesta inmune muy coordinada tras la infección con IHNV en un órgano hematopoyético como lo es el riñón anterior; Esta respuesta es llevada a cabo por el sistema inmune del hospedador, y probablemente se solapen resultados debidos a estrategias empleadas por el virus para lograr su replicación y supervivencia.

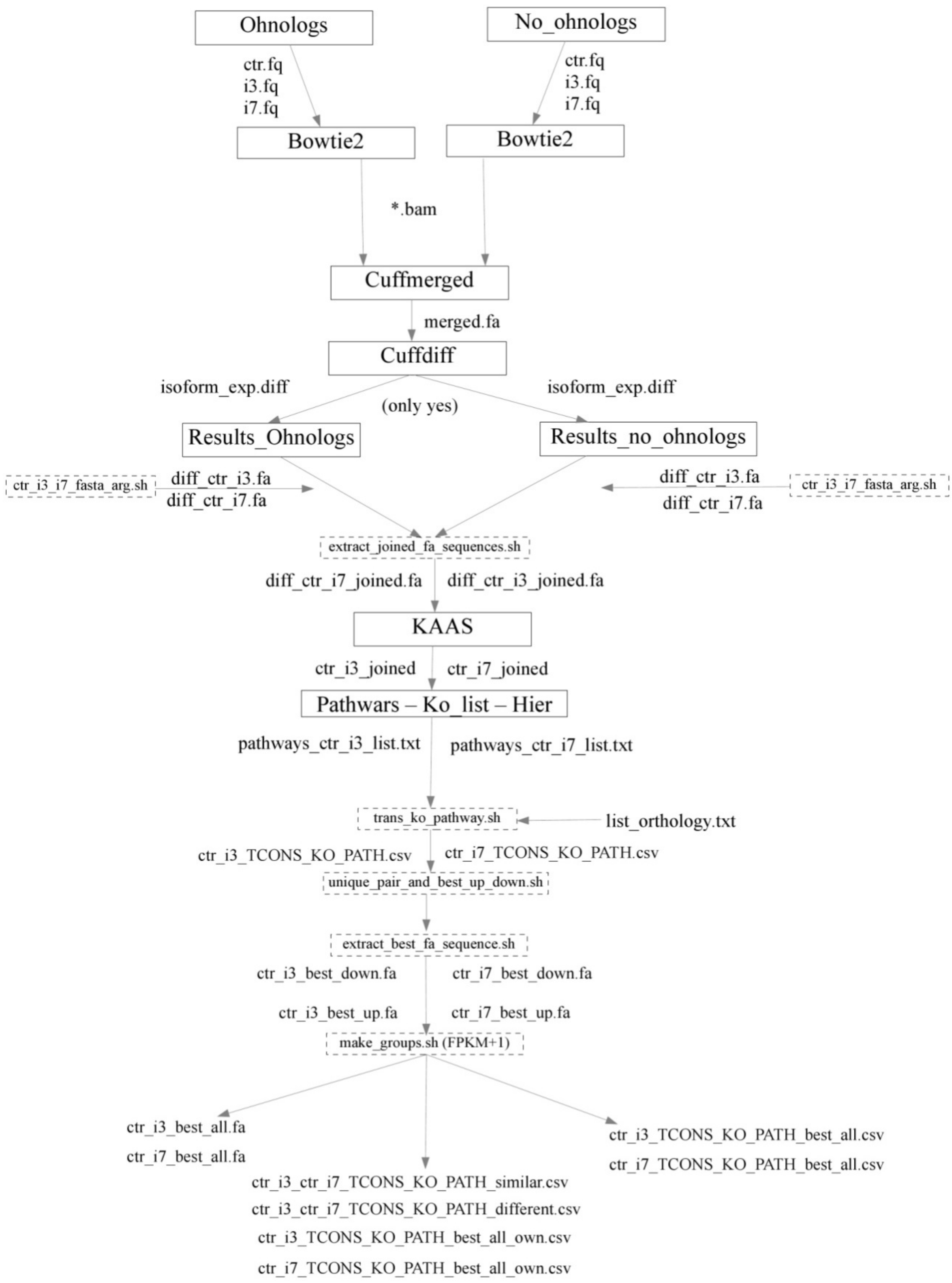


Figura 12: Esquema del procedimiento del análisis de los datos de RNA-seq.

RNA-seq profiles from rainbow trout kidney tissue after infectious haematopoietic necrosis virus (IHNV) infection at early (asymptomatic) and symptomatic time points.

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The rhabdovirus infectious hematopoietic necrosis virus (IHNV) is the etiological agent of a severe viral disease that causes systemic infection and significant mortality in a variety of salmonid fish. Currently, there is only limited information on the host cell response to the virus. This work characterizes the transcriptional response of rainbow trout *Oncorhynchus mykiss* to IHNV infection by conducting a high throughput RNA-seq analysis using Illumina next-generation sequencing. RNA-seq was performed on the anterior kidney tissue of rainbow trout at two time points post-infection that represent different stages of the disease. Duplicate gene isoforms from orthologous references and unique genes from non-ohnologous isoforms were then combined and reported as the total transcripts. Once the isoforms were combined, a total of 6875 differentially expressed transcripts (DETs) and 5857 DETs were identified in response to IHNV at 3 and 7 days post-infection (dpi.), respectively, compared with uninfected control fish; 3141 of the DETs were common to the fish analyzed at each time point. A total of 340 DETs were found in 3 dpi. and 7 dpi. samples with a fold change > 2 or < 0.5 relative to control fish. At least one annotated GO term could be assigned to most of the DETs (173 out of 340).

The filtered DETs were classified into transcripts involved in events at both 3 and 7 dpi. (47 DETs) and transcripts that were modulated at just one of the post-infection time points (198 DETs up-regulated at 3 dpi. and 18 at 7 dpi.). In addition, functional annotation by KEGG Orthology rendered 58 pathways that contain modulated genes at both 3 and 7 dpi., 170 pathways activated uniquely at 3 dpi. and 34 at 7 dpi. of these, TLR, JAK-STAT and apoptosis pathways were further examined, and the highlighted up- or down-regulated genes were graphically represented. In addition, interesting immune pathways, such as the chemokine signaling pathway, hematopoietic cell differentiation, cytokine-cytokine receptor interaction, the TNF signaling pathway, and natural killer cell mediated cytotoxicity, are shown in the supplementary materials.

Keywords: RNA-seq, infectious hematopoietic necrosis virus, IHNV, IHNV transcriptional profiles, fish viruses

I. INTRODUCTION

The production of salmonid fish is widely distributed, and rainbow trout (*Oncorhynchus mykiss*) is one of the continental species for which culture has been economically successful. However, disease outbreaks are being increasingly recognized as a significant constraint to fish production because large numbers of animals can be rapidly infected and die. Infectious hematopoietic necrosis (IHN) is a severe viral disease of farmed salmonids. The disease is caused by infectious hematopoietic necrosis virus (IHNV), an aquatic rhabdovirus of the genus Novirhabdovirus (Van Regenmortel, M.H.V Fauquet, Bishop, Editors, 2000). IHNV is a non-segmented negative strand RNA virus and is responsible, in large part, for the increased losses in the US salmonid fish industry. IHN occurs on farms rearing fry or juvenile rainbow trout in freshwater where acute outbreaks can result in very high mortality. In Europe, rainbow trout is the most affected fish species. The virus causes asymptomatic infections in adult salmonids but can cause mortalities of up to 100% in young salmonids, depending on the virus strain and environmental conditions; moreover, smolts can develop clinical disease within 7 days of being introduced to seawater. Clinically affected fish are lethargic or may have whirling behavior and, among other symptoms, have cranial swelling (cephalic bumps), abdominal distension, exophthalmia, and darkened skin (Yasutake Amend, 1972).

Salmonid fish have been the subject of a large number of studies on infectious diseases and the protective immune mechanisms mounted in response to viral infections, particularly those directed towards the development of vaccines against rhabdoviruses. The first approaches, more than 25 years ago, provided the basis for future DNA vaccines against IHNV. Since then, the progressive improvement of methods, resources and technology have provided a better understanding of viral pathogenesis and the basic features of the immune response to viral infections in fish.

Most studies have used microarray technology and reported interesting results, many of which are related to IHNV (Purcell, Marjara, Batts, Kurath, Hansen, 2011). The development of techniques such as high-throughput RNA sequencing can provide a comprehensive assessment of RNA expression profiles with advantages including high-throughput sequencing data acquisition, low background, high sensitivity and reproducibility. RNA-seq has been used in rainbow trout to study the expression levels of nucleated erythrocytes (Morera *et. al.*, 2011), to identify DNA markers associated with growth (Salem *et. al.*, 2012) and to identify differentially expressed transcripts in response to muscle effort (Palstra *et. al.*, 2013). However, RNA-seq has not yet been applied to fish virus studies. Characterizing responses to the pathological changes induced by viral infection at the transcriptome level offers the opportunity to understand the underlying basis for illness and associated physiological stages.

In the current work, a high-throughput deep sequencing-by-synthesis technology (RNA-seq) approach was used to perform a deep transcriptome analysis of fry rainbow trout infected with IHNV. Salmonid fishes are descended from a whole genome duplication event in an autotetraploid ancestor (4R), which is estimated to have occurred 88-103 million years ago (MYA). The genomes of these species have a tendency to return to a diploid state, but there is no evidence of complete diploid restoration because extensive rearrangements among chromosomes as well as tetrasomic inheritance in males has been observed. Whole genome duplication is the process responsible for the rapid diversification and evolutionary innovation of the species of the *Oncorhynchus* genus (Berthelot *et. al.*, 2014). Therefore, we used ohnologous (paralogous genes that have originated from a process of whole-genome duplication) and non-ohnologous sequences from (https://www.genoscope.cns.fr/spip/) as the reference for the RNA-seq data analysis, which constitutes a qualitative resource to represent the maximum number of unique and duplicated transcripts in RNA-seq results. Finally, the kidney was chosen for these studies because of the key role it plays as both a hematopoietic and viral replication target organ.

The present study aims to provide an approach to examine the transcriptional events induced in trout after infection and to establish a comprehensive overview of the transcriptome response and pathological changes that occur at two time points after IHNV infection (3 and 7 days post-infection, dpi.). Such knowledge will increase our understanding of the immune response in rainbow trout and provide new approaches to prevent infection and facilitate vaccine design. To this end, we identify key candidate genes involved in the important biochemical pathways on which selection is likely to act during viral infection. The RNA-seq approach aids in the identification of genes with altered expression due to IHNV infection. Additionally, pathways responding to IHNV infection were identified through KEGG Orthology and gene ontology analyses. The results presented here are some of the first applying this technology to a fish virus and are focused on the viral effects induced in rainbow trout by IHNV.

II. MATERIALS AND METHODS

A. Ethics statement

The experiments described comply with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals and were previously approved by The Consejo Superior de Investigaciones Cientficas (CSIC) Ethics Committee.

B. Experimental fish: rainbow trout

Rainbow trout (with mean weight and size of 3 g and 8 cm, respectively) were purchased from a local spring water farm with no history of viral disease, and transported live to Centro de Investigaciones Biolgicas (CSIC, Madrid, Spain). The fish were maintained in a rearing facility and acclimatized at a temperature of 15C for a period of 15 days prior to the start of the experiments. The trout were kept under a 12/12 h light/dark regime in 350 L closed recirculating water tanks (Living Stream, Frigid Units Inc., Ohio). No fish showed any clinical symptoms. In addition, two pools of 5 fish each were tested by standard methods to confirm the absence of IHNV or any other salmonid virus by isolation using EPC cells. Groups of trout were maintained at 141 C in separate 45 L aquaria supplied with non-chlorinated water using exterior carbon filters (Eheim, Madrid, Spain) and additional aeration. The trout were fed a daily diet of commercial pellets. The water-quality parameters were equal and maintained at optimum levels in all tanks.

C. Virus propagation

The IHNV strain obtained from the American Type Culture Collection (ATCC VR-714) was propagated in the BF-2 cell line from bluegill fry (Lepomis macrochirus, ATCC CCL-91) in Leibovitzs medium (L15, Gibco, Spain) supplemented with 100 IU/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine and 2% fetal bovine serum (Gibco, Spain) at 14$\pm$1°C. Supernatants from infected BF-2 cell monolayers were clarified by centrifugation at 1000 g for 20 min after extensive cytopathic effects were observed. Clarified supernatants were used for the experiments. Viral titration was performed in 96-well culture plates. The infective titer was determined as the 50% tissue culture infective dose ($TCID_{50}$/ml).

D. IHNV challenge by immersion

The trout were divided into 2 groups of 25 trout each. Group 1 was infected with IHNV (VR-714 strain obtained from the American Type Culture Collection) in a reduced volume of water for 2 h with aeration (3×10^5 $TCID_{50}$/ml of IHNV). Group 2 was handled similarly but was mock infected. At 3 and 7 dpi., the trout were anaesthetized by immersing in 50 mg/ml tricaine-ethanesulfonate (MS-222, Sigma, Madrid, Spain) buffered in PBS prior to handling. After decapitation, the head kidneys were harvested from each trout. The organs were immediately immersed in RNAlater (Ambion, Austin, USA) and kept at 4°C overnight before being frozen at -70°C until processed. There were 6 trout for each time point.

E. Isolation of total RNA and cDNA synthesis

Upon completion of the IHNV infection trial, fish were sacrificed and dissected immediately to remove their kidneys. We selected the kidneys due to previous research linking this tissue type to immune responses analyzed by microarrays (Ballesteros, Saint-Jean, Encinas, Perez-Prieto, Coll, 2012). The head kidneys were individually homogenized using the Tissue Lyser Cell Disruptor (Qiagen, IZASA SL, Madrid, Spain) for 5 min at 50 Hz with 2 mm glass beads. Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions, and the concentration and purity of the RNA obtained were measured in a NanoDrop spectrophotometer. RNA integrity and quality were measured in an Experion Automated Electrophoresis System (Bio-Rad, Madrid, Spain). The RNA integrity number (RIN) was calculated for each sample, and only the best quality RNAs (RIN number ≥ 9) were processed.

F. RNA extraction, Illumina library preparation and sequencing

Equal amounts of total RNA pooled from six fish in each group were used to prepare mRNA libraries for sequencing following the standard Illumina protocol. Library preparation and sequencing processes were performed in the Parque Cientifico de Madrid (Campus de Cantoblanco, Madrid, Spain). The RNA-seq libraries were sequenced with a read length of 75 nucleotides with single-end reads in two different lanes of an Illumina GAiix instrument. To increase the read coverage and examine the technical sequencing variation, a total of two sequencing runs were performed.

G. RNA-seq analysis

For quality control, raw sequence reads were sorted by individual samples, and adapters were trimmed by the sequencing service provider prior to analysis. Quality control was performed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). Based on the FastQC quality statistics, the reads had adequate and consistent scores that were >35. The sequence reads for each sample were mapped back to the assembled transcriptome divided into two different parts consisting of the ohnologous and non-ohnologous genes (Berthelot *et. al.*, 2014); the alignment approach was tested using BWA, Bowtie and Bowtie2 tools (data not shown) (Langmead Salzberg, 2012; Langmead, Trapnell, Pop, Salzberg, 2009; Li Durbin, 2009). The best alignment results were obtained using Bowtie2 (Langmead Salzberg, 2012). Bowtie2 was implemented in the -k alignment mode with the maximum number of alignments set to k=1 for reads mapped uniquely to the reference sequence (non-ohnologous), and k=6 for mapping to duplicated genes (ohnologous) allowing for a maximum of two mismatches. The Bowtie2 alignment files were then used as input for the Cufflinks2 program to obtain assembled transcripts. The expression score of each transcript was represented in fragments per kilobase of exon per million fragments mapped. I3 and I7 samples from fish infected at 3 and 7 dpi., respectively, were compared with the control fish group. Finally, pairwise comparisons of the I3/Control and I7/Control groups were performed with the Cuffdiff module (Trapnell *et. al.*, 2013). Cuffdiff tests the statistical significance of each observed change in expression between pairs by using the log of the ratio of expression under the two conditions. As the data are approximately normally distributed, Students t-test was used to find transcripts that were differentially expressed. In this approach, the variance in fragment counts is modeled by assuming a negative binomial distribution. As a result of the t-test, a p value is obtained and adjusted for multiple testing by the Benjamini Hochberg method, giving a final false discovery rate (FDR). Transcripts with an FDR$<$ 0.05 were considered to be differentially expressed between conditions.

H. Functional annotation and pathway analysis of the differentially expressed transcripts (DETs)

DETs were annotated using two different languages: the Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) by Orthology (KO) systems. The GO analysis consistently describes gene products in all eukaryotes in three different categories: molecular function, biological process and cellular component (Ashburner *et. al.*, 2000). The KO is an annotation of orthologous genes from all available genomes based on pathways. Thus, each KO entry represents a group of orthologous genes that are associated with a gene product in the KEGG pathway diagram (Moriya, Itoh, Okuda, Yoshizawa, Kanehisa, 2007).

The GO functional annotations were performed by using the Blast2GO software (Gtz *et. al.*, 2008). This program uses BLAST to find sequences similar to the DETs and subsequently maps the BLAST hits obtained to associated GO terms. Finally, applying an annotation rule, it selects the GO pool obtained in the previous step. For the BLAST annotation, the BLASTX algorithm was used with the minimum E-value score set to 10^{-6} and the number of BLAST hits set to 5. For GO annotation, the annotation rule was applied with default parameters. Significantly up- and down-regulated transcripts were selected and blasted against the NCBI database using BLASTX in the program Blast2GO. BLASTX was performed against the NCBI nucleotide database with the minimum E-value score set to 10^{-10}. To assign gene ontology terms to each annotated sequence, successful blast hits were mapped and annotated using Blast2GO for the entire assembled transcriptome with the annotation cut-off threshold set to 55 and the GO level weighting set to 2.

The KAAS web-server (Moriya *et. al.*, 2007) on the KEGG website was used to annotate each DET to a KO identifier. As with Blast2Go, the process starts with the BLAST algorithm finding homologous genes in the reference sequence set(s) that we defined. Among these homologous genes, orthologs are defined according to a bi-directional hit rate, and the final KO is assigned by a score based on the likelihood and the heuristics for each KO term. The KAAS also provides a list of pathways linked to the KO terms and their graphical maps.

I. Validation of gene expression fold changes by reverse transcription quantitative polymerase chain reactions (RTqPCR)

Seven transcripts covering the full range of gene expression fold changes were selected for RTqPCR. The PCR primers are listed in supporting information Table S1. Aliquots from pools of RNA samples used for RNA-seq were tested in duplicate for each gene. The cDNA synthesis was performed with 5 μg of the RNA primed with oligo-d(T) (25 pmol/μl). The Super Script II kit (Invitrogen, Spain) was used for reverse transcription. The cDNA was diluted 1:4 in DEPC-treated water, and 1 μl of the diluted cDNA was used for each real-time PCR assay reaction.

J. Quantitative estimation of transcripts for selected immune-related genes by RTqPCR

The RTqPCRs were performed using the SYBR® green method, in an iQ5 iCycler thermal cycler (Bio-Rad Laboratories, Inc., Madrid, Spain). The qPCR amplifications were performed in 96-well plates by mixing 1 μl of 4-fold diluted cDNA, 12.5 μl of 2× concentrated iQ SYBR® Green Super mix (Bio-Rad), 0.3 μM forward primer and 0.3 μM reverse primer in a 25 μl reaction volume for each sample. The thermal profile was 10 min at 95°C, followed by 40 amplification cycles of 10 s at 95°C, 1 min at 60°C and a dissociation cycle (1 min at 95°C and 1 min at 60°C). After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification. No amplification product was observed in controls containing no RNA samples. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) and the relative quantification of the amplified gene products was calculated using the comparative Ct method. The elongation factor 1α (ef1a) was used as a housekeeping gene in each RNA sample to normalize the results for variation in mRNA and cDNA quantity and quality (normalized values). All of the qPCR reactions were performed in duplicate (technical replicates), and their mean Ct values were used for the calculations. First, the Ct for each gene was normalized to the corresponding ef1a C_t ($\Delta Ct_{\mathrm{gene}} = Ct_{\mathrm{gene}} - Ct_{\mathrm{ef1a}}$). Second, mean control Ct values (mean $\Delta Ct_{\mathrm{control}}$) from 3 trout without any treatment or mock infected for the IHNV infection were calculated. Third, fold changes in concentration were calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct_{\mathrm{gene}} = \Delta Ct_{\mathrm{gene}} - \Delta Ct_{\mathrm{control}}$. Finally, the mean and standard deviation for each gene from 3 trout were calculated and presented (User Bulletin #2: ABI PRISM 7700 sequence detection system, PE Applied Biosystems).

K. Statistical analysis for RTqPCR

Prior to statistical analyses, the normal distribution of the data were checked and confirmed using the ShapiroWilk test. Data are presented as the mean ± standard deviation of 6 trout. Factorial ANOVAs were run to determine whether the expression level of the differentially expressed gene differed between the infected and the control fish groups. All statistics were run in SPSS Version 16. The p value, which was less than 0.05, was considered significant.

III. RESULTS AND DISCUSSION

A. Experimental IHNV infection in the rainbow trout

The sequential progression of IHNV infection in tissue was first described by classical immunohistochemical analysis (Drolet, Rohovec, Leong, 1994). Negative results were found at the first dpi. (pre-epizootic period), and a progression to positive results was seen after 5 dpi. in hematopoietic tissues, such as the kidney, and in the gastrointestinal organs. Six or seven days after exposure to the virus, several signs of disease appeared, and the epizootic period became apparent. The associated disease produces acute hemorrhagic septicemias that impact multiple organs and lead to the activation of several characteristics events. A large amount of the virus was produced in the kidney, where the strongest and most persistent infection was detected. Together with the spleen, the kidney is one of the primary sites of infection and inflammation and is therefore an adequate target organ to analyze the rainbow trout response to IHNV by next-generation sequencing. In our case, the fish were asymptomatic at 3 dpi., but at 7 dpi., the fish showed

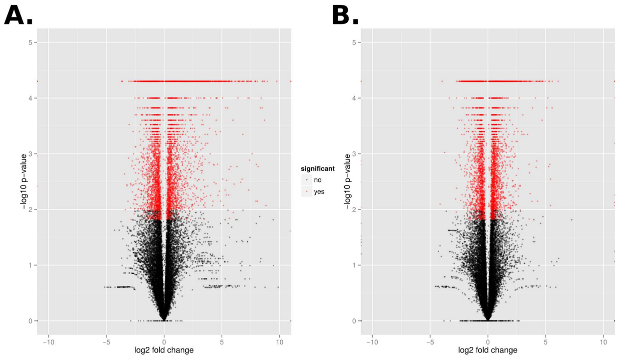

FIG. 1: Differentially expressed transcripts (DETs) in the head kidneys of rainbow trout at 3 days (A) and 7 days (B) post infection (dpi.) with IHNV. The results are shown as the log2 fold change in expression (control fish/infected fish) versus the log10 of the p-value. Red dots above zerofold change represent significantly up-regulated DETs, whereas red dots below zerofold change represent significantly down-regulated DETs at the 0.5 false discovery rate. Black dots represent not significantly DETs.

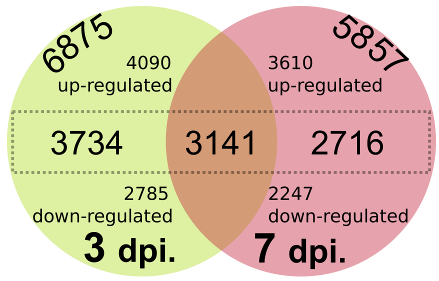

FIG. 2: Overview of transcriptional data in the head kidney of IHNV-infected rainbow trout. Venn diagram indicating the number of differentially expressed transcripts (DETs) across two key comparisons (initial asymptomatic IHNV infection at 3 dpi. vs. non-infected (control) fish) and symptomatic infection at 7 dpi. vs. control fish. The overlap between common up- and down-regulated DETs from both asymptomatic and symptomatic IHNV infection is shown in the marked center of the figure, while the number of DETs exclusively expressed at 3 and 7 dpi. are denoted on the left and right, respectively.

clinical signs of disease, e.g., exophthalmia, hyperpigmentation, corkscrew swimming, anorexia, and death (Amend Smith, 1974). The mortality rate rapidly increased between 7 and 12 dpi. and reached 100% cumulative mortality at 20 dpi. (data not shown).

B. Sequencing and mapping

To characterize the response of rainbow trout to IHNV infection, a high throughput analysis based on the RNA-seq data was performed. We sequenced three libraries: kidney tissue control group (from nave fish), kidney tissue from infected fish sampled at 3 dpi. (initial or asymptomatic fish) and fish sampled at 7 dpi. (symptomatic infection). In total, all of the lanes of the Illumina GAiix Format 1x75 produced close to 8 million single-end reads (1 × 75 bp), and the data were deposited in the SRA database.The number of reads in each group was well balanced, with 16, 122, 108 reads from infected fish at 3 dpi., 16, 378, 765 reads in the infected fish sampled at 7 dpi. and 16, 192, 950 reads in the non-infected control fish group. Approximately 6, 361, 930 (38.99%) of these reads were mapped uniquely to the non-ohnologous gene reference, and 2, 109, 765 (12.93%) of the reads were mapped as duplicates to the ohnologous gene reference (Table 1). The level of gene expression was then analyzed by calculating the number of unambiguous reads for each gene and normalizing it to the number of RPKM (Trapnell *et. al.*, 2013).

C. Identification of DETs in response to initial IHNV infection and symptomatic IHNV infection

RNA-seq was performed at two time points post-infection that represented different stages of disease. Duplicate gene isoforms from orthologous references and unique genes from non-ohnologous isoforms were then combined and reported as the total transcripts. Once the isoforms were combined, a total of 6875 DETs in response to asymptomatic or initial IHNV infection and 5857 DETs involved in symptomatic IHNV infection were identified in comparison with the control fish group; 3141 of these transcripts were common to the fish analyzed at both 3 and 7 dpi.. For more information, see supporting information Table S2. Only 2785 transcripts were down-regulated with a range of fold changes from -3.6819 to -0.0484, and the remaining 4090 were up-regulated with a range of fold changes from 0.07 to 9.75 in the case of initial or asymptomatic IHNV infection (Figure 1A). A total of 2247 DETs were down-regulated with a range of fold changes from -4.13 to -0.079, and the remaining 3610 transcripts were up-regulated with a range of fold changes from 0.049 to 6.08 in the case of symptomatic IHNV infection (Figure 1B).

When comparing the transcriptional profiles recorded in rainbow trout 3 and 7 dpi. with those of the control fish group, the percentage of up-regulated transcripts were higher than the percentage of down-regulated transcripts, which could be related to the progression of IHNV infection. Despite the higher DETs found at 3 dpi. compared with 7 dpi., the virus seems to modulate the expression of several genes, which showed increased differential expression at 7 dpi. when compared to 3 dpi. (59.49% up-regulated genes at 3 dpi. compared to 61.63% at 7 dpi., n=4090 and n=3610, respectively). In contrast, most of the differentially decreased DETs were found at 3 dpi. in comparison with 7 dpi. (40.5% down-regulated genes at 3 days compared to 38.36% at 7 days, n = 2785 and n=2247, respectively) (Figure 2).

D. Functional annotation and pathway analysis

Initially, only the 340 DETs found at 3 dpi. and 7 dpi. with a fold change > 2 or < 0.5 compared to control samples were functionally annotated to allow for a clear inspection of the main differentially expressed genes during the initial or asymptomatic infection and symptomatic infection. Further, IHNV interacts with its host at multiple stages during disease development. Thus, these filtered DETs were classified according to the following conditions: a) transcripts that were present in both samples, i.e., genes whose temporal expression patterns differentiate the response of asymptomatic from symptomatic fish, and b). transcripts that were present in just one sample, i.e., genes uniquely involved in the temporal dynamics of IHNV infection (genes exclusively implicated in events at 3 or 7 dpi.).

1. *Transcripts that were present in the fish sampled at 3 and 7 dpi.*

The analysis allowed us to identify 32 differentially expressed genes at both 3 and 7 dpi. (modulated genes), most of which were up-regulated (n=28), and the remaining 4 DETs were down-regulated. In the kidneys of the infected rainbow trout, the range of fold changes in modulated genes varied from -2.69 to 8.4 at 3 dpi. and from -2.2 to 5.5 at 7 dpi.. The KO term that was assigned to each DET is shown in Figure 3A, most of the up-regulated KO terms had fold changes that varied from 2 to 6.5 (∼80% of n=25). Among the 32 DETs that were found at 3 and 7 dpi., a selection of those that showed high and differential up-regulation at these time points were also analyzed. The KOs with the highest fold changes during IHNV infection were K09447 (IRF7), K04519 (IL1B) and K14754 (Mx1). These genes are related to host defense and the induction of cellular antiviral stages. In addition, K06708 DET (CD273 or programmed cell death 1 ligand 2) was implicated in the cell adhesion molecule (CAM) pathway and K10352 (MYH or myosin heavy chain) was involved in the tight junction pathway, which is a type of cell-cell junction that controls the paracellular permeability across the lateral intercellular space and maintains cell polarity. Alterations in the expression or localization of tight junction proteins have been described in several human neurological disorders.

The genes with the highest expression levels (fold-change relative to the control fish) corresponded to the 3 dpi. time point in comparison to 7 dpi., thus indicating a time-dependent induction of particular genes during the initial phases of infection. Among these highly expressed genes, five showed changes ranging from 2 to 8-fold, and they were all related to antiviral functions; RSAD2 and Mx-1 were the most significantly up-regulated genes upon IHNV infection (up-regulated 8-fold, Figure 3A).

The radical S-adenosyl methionine domain-containing protein 2 (RSAD2) gene from mammals is related to the viperin antiviral protein, which is induced by cytomegalovirus infection in mammals. In fish it is known as vig-1, and it was up-regulated and identified in rainbow trout after infection with the rhabdovirus viral hemorrhagic septicemia virus (VHSV) (Boudinot, Massin, Blanco, Riffault, Benmansour, 1999). Mx-1 is an antiviral protein induced by IFN, and it has been shown to protect cells against a variety of fish viruses, such as IPNV and IHNV (Larsen, Rokenes, Robertsen, Rkenes, 2004).

Other highly expressed genes at 3 dpi. vs. 7 dpi. (6-fold) were the following: the TNFRSF6B gene (tumor necrosis factor receptor superfamily member 6B), a receptor involved in the cytokine-cytokine receptor pathway; the TNF superfamily, which plays a key role in inflammation, host defense, autoimmunity, organogenesis, cellular apoptosis, and differentiation and fulfils its functions by interacting with their respective receptors (TNFRs); USP18 (ubiquitin carboxyl-terminal hydrolase), which is a key regulator of the interferon-driven gene network modulating pancreatic beta cell inflammation and apoptosis; IRG-1 (aconitate decarboxylase), which has been associated with metabolic and immunity reactions mediated by itaconic acid (Michelucci *et. al.*, 2013); and IRF 7 (interferon regulatory factor 7), which is crucial in regulating the type I interferon (IFN) response to viral infection. IRF 7 activation upon TLR 7 signaling precedes binding of the activated molecule to the promoter region of type I IFN for its synthesis (Holland *et. al.*, 2008).

CXCL11 (up 6-fold) is another IFN induced gene that functions as a chemokine and is the dominant ligand for CXC receptor 3 on immune cells. Its release initiates a chemotactic response in activated T cells recruiting them to lymphoid or infected organs. Finally, interleukins are molecules involved in the intercellular regulation of the immune system. IL1 was one of the few cytokines discovered in fish, including salmonids, by homology cloning (Secombes, Wang, Bird, 2011); the up-regulation observed here correlates with viral infectivity and innate immune responses most closely related to IFN. The profiles of expressed genes agree with the events that take place in the host after viral infection. Infections with bacteria or stimulation with poly I:C also raised IL1β levels.

To differentiate those transcripts that are modulated at 3 dpi. and 7 dpi. from those transcripts that are similarly expressed during the infection, the first quartile of the absolute differences in the fold changes was considered as the cutoff value. In this way, the cutoff value was set at an approximate value of 0.1. Therefore, DETs with a difference of expression under 0.1 were considered to be evenly expressed and those with a difference of expression above this value were treated as modulated or differentially expressed. In relation to the similarly expressed genes, the remaining 13 DETs were assigned to KO terms, and they are shown in Figure 3B where we can see that most of them (n=7) were up-regulated with a range of fold changes from 2.12 to 3.32. The remained six

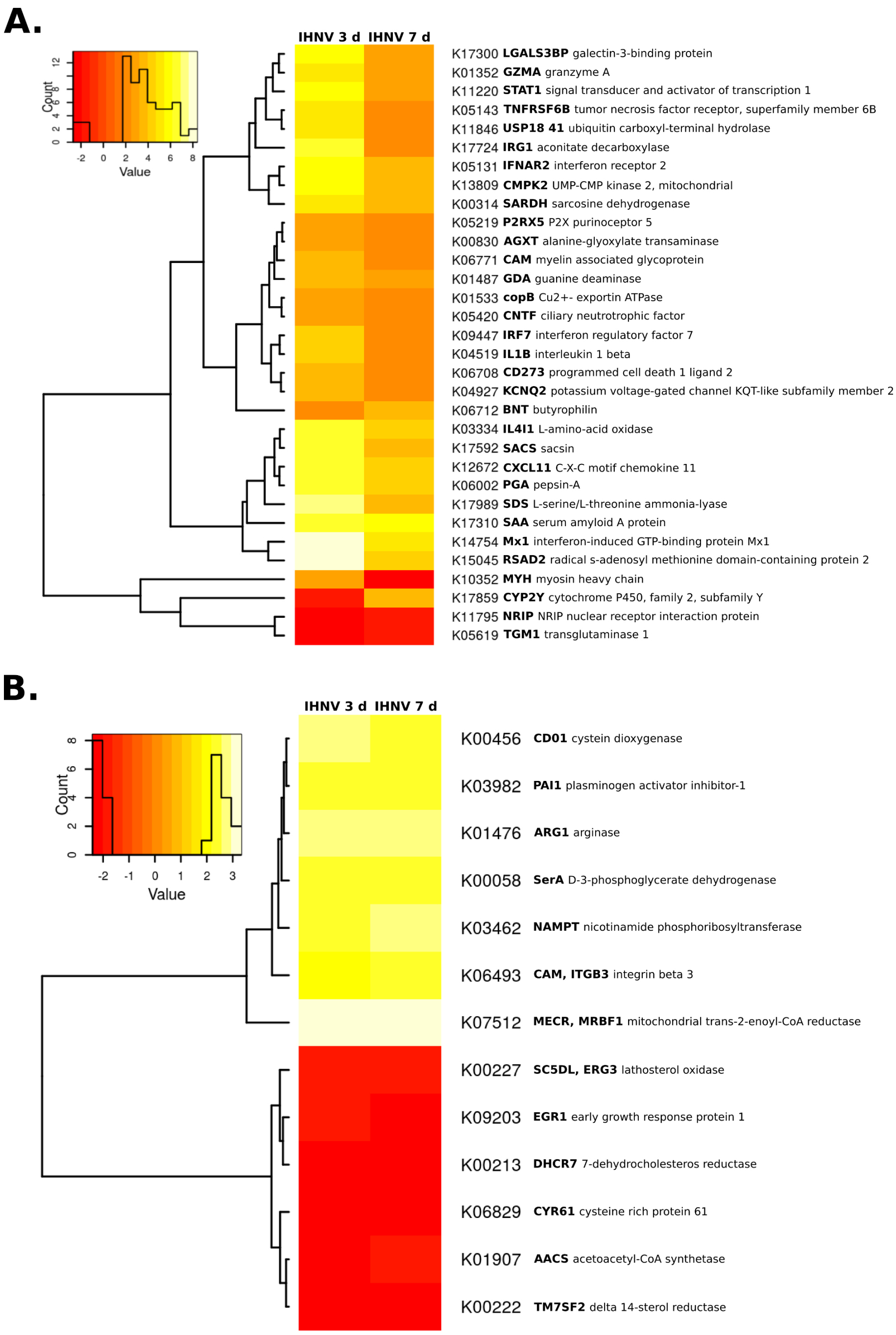

FIG. 3: Unsupervised hierarchical clustering and expression heat map of differentially regulated transcripts (DETs) from the head kidneys of rainbow trout at 3 (A) and 7 (B) days post-IHNV infection (dpi.) relative to uninfected fish (control). Organs pools from six fish were analyzed. The x-axis contains the expression data from experimental groups (each column represents the data at 3 and 7 dpi.) and the y-axis represents the fold expression of the DETs. Only genes that were differentially expressed in pairwise T-tests at a P<0.05 are shown. The scale bar in the upper right corner ranges from white (up-regulated) to red (down-regulated) with the degree of intensity indicating the degree of fold change.

DETs were down-regulated with fold changes ranging from -1.82 to -2.40. Among the up-regulated KOs, we found k00456 CD01 (cysteine dioxygenase), which is involved in the cysteine and methionine pathways (amino acid metabolism), and PAI1 (serpine) plasminogen activator inhibitor-1. PAI1 is an important component of the coagulation system, the primary inhibitor of tissue- and urokinase-type plasminogen activators and is considered a critical regulator of the fibrinolytic system; its function has been described in osteosarcoma cells where it stimulates the assembly of the fibronectin matrix and integrins (Vial McKeown-Longo, 2008). Previously, Bearzoti *et. al.* (1999) established that fibronectin is the cell receptor for fish rhabdoviruses (Bearzotti *et. al.*, 1999), and its up-regulation may favor the early steps of cellular viral infection. Our RNA-seq results show that PAI1 levels are increased by approximately 3-fold at both 3 and 7 dpi.. The results are consistent with those of Miller *et. al.* (2007) who found in IHNV infections up-regulation of the uPAR gene that, in its turn, increases concentrations of fibronectin in cellular membranes (Miller *et. al.*, 2007). The authors suggested an IHNV-induced activity to facilitate viral entry.

Another up-regulated gene during IHNV infection was ARG-1 (arginase). The arginase enzyme metabolizes L-arginine to L-ornithine plus urea and has a fundamental role in the hepatic urea cycle, but a role has also recently been described in the human and murine immune systems. In mammals, arginase is induced by Th2 cytokines and inflammatory agents that participate in a variety of inflammatory diseases by down-regulating nitric oxide synthesis and inducing fibrosis and tissue regeneration (Munder, 2009). In humans, arginase is liberated during inflammation. Thus, arginase up-regulation in infected fish kidney is consistent with inflammatory processes, as a large amount of IHNV is produced in hematopoietic tissues with corresponding damage.

Significant down-regulation of some DETs was detected in both 3 and 7 dpi. samples. Examples include KOs k00222 (TM7SF2-delta14-sterol reductase) and k00213 (DHCR7-7-dehydrocholesterol reductase), which have been implicated in cholesterol biosynthesis as part of the lipid metabolic pathway, and k06829 (CYR61-cysteine rich protein 61 gene), which is growth factor-inducible and promotes endothelial cell adhesion.

2. Transcripts that are present exclusively in either the 3 or 7 dpi. samples

Genes that are differentially expressed only at 3 dpi. or 7 dpi. were analyzed separately. At 3 dpi., we found 198 DETs with fold changes ranging from -2.9 to 9.3, with 165 up-regulated (fold changes from 1.66 to 9.31) and 32 down-regulated (fold changes from -2.92 to -1.54). Figure 4A shows up-regulated KOs with fold changes >3, n=31. Cytokines are a family of low molecular weight proteins secreted by activated immune-related cells upon induction by viruses and other pathogens. Our present results show that the progression of IHNV infection induces the differential expression of many types of genes that are time-dependent; several of these were cytokines that were up-regulated 3 to 5-fold at 3 dpi. but not at 7 dpi.. As the elimination of pathogens requires the recruitment and activation of macrophages, neutrophils and lymphocytes to the infected tissues (Savan Sakai, 2006), several representative genes associated with innate immunology were detected during the early stage of IHNV infection.

Some examples include CXCR3, LTA, IL11, IL6 and IL12A. CXCR3 is a well-studied G protein receptor in higher vertebrates that has scarcely been examined in fish. This gene is highly expressed in effector T cells and plays an important role in T cell trafficking and function. In murine models, CXCR3 and its ligands regulate the migration of Th1 cells into sites of Th1-driven inflammation. In different animal models, the expression of this gene in effector T cells grants them entry into otherwise restricted sites and has been shown to be related to viral diseases producing inflammation (Groom Luster, 2011).

In fish, CXC chemokines are highly distributed in hemopoietic organs such as kidneys because these tissues contain greater numbers of defense cells, such as lymphocytes and macrophages. Our results on the up-regulation of CXCR during the early stage of IHNV infection may be explained by the initial process of infection; it has been reported that the kidney is infected with virus from the capillaries surrounding the glomeruli and renal tubules; virus enters the tubules, buds out of the basement membrane and productively infects the adjacent hematopoietic tissue (Drolet *et. al.*, 1994). Alejo and Tafalla (2011) reported that in fish, the CXC chemokines and their receptors play a major role in the management of leukocyte transport from the blood into the infected cells during inflammation. Another gene implicated in this process was LTA (or TNFB), a lymphotoxin alpha belonging to the TNF superfamily that is involved in processes regulating proliferation, survival, apoptosis and pathways, such as cytokine-cytokine interaction, NF-kappa B signaling and TNF signaling. It has also been described in human viral infections, such as herpes virus and HTLV infection.

DHX58 was another of the genes uniquely expressed at 3 dpi.; it is an ATP-dependent RNA helicase gene also known as RIG-I-like receptor 3 (RLR-3) or RIG-I-like receptor LGP2 (RLR). It is involved in antiviral signaling and triggers a signal for innate antiviral responses, including the production of type I IFN (RIG-I and MDA5) (Yoneyama *et. al.*, 2005).

In innate immunity, macrophages can secrete cytokines including IL1, IL6, IL12, and TNF-α, which are indispensable for macrophage, neutrophil, and lymphocyte recruitment to the infected tissues and their activation as pathogen eliminators. Our RNA-seq analysis reported up-regulation of all these ILs except for IL1 in the infected trout only at 3 dpi., showing 3-5-fold increased expression levels.

IL6 is considered a proinflammatory interleukin, and it is released during the cytokine cascade following bacterial infections in rainbow trout (Costa, Maehr, Diaz-Rosales, Secombes, Wang, 2011). IL11 also belongs to the IL6 family of cytokines. IL11 is a multifunctional cytokine that stimulates hematopoietic progenitor cells and exerts a series of important immunomodulatory effects. The Teleost fish IL11 orthologue was first reported in trout and is widely distributed and modulated by infection and other cytokines, suggesting that fish IL11 is an active player in the cytokine network and the host immune response to infection (Wang, Holland, Bols, Secombes, 2005).

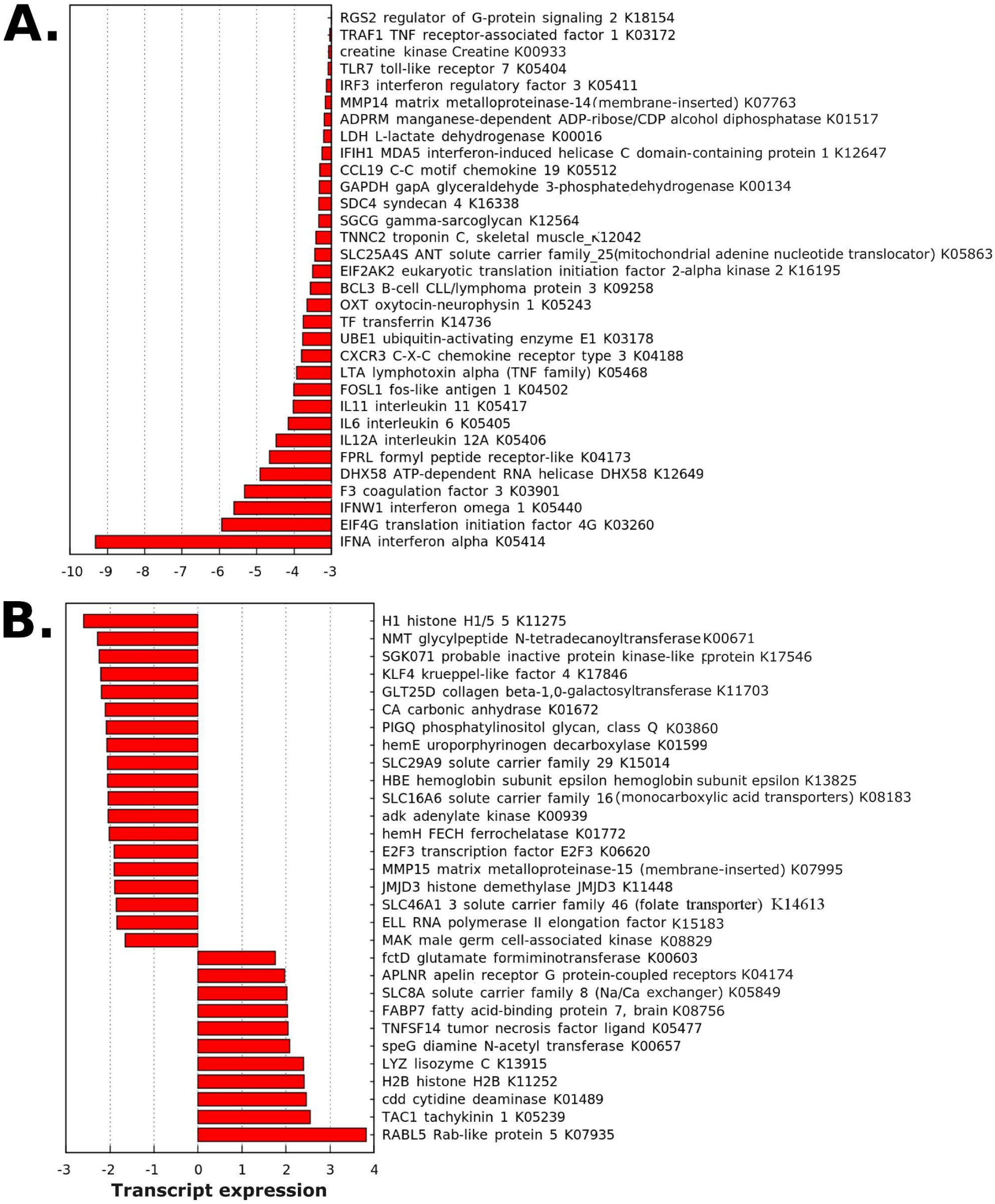

FIG. 4: Histograms with fold changes in differentially expressed transcripts (DETs) in the head kidneys of *O. mykiss* exclusively expressed at (A) 3 dpi. and (B) 7 dpi. in comparison with uninfected fish (control). Organ pools from six fish were analyzed. The y-axis contains the expression data from each day of IHNV infection and the x-axis represents the fold expression of the significant genes. Only the genes that were differentially expressed in pairwise T-tests at a P<0.05 are shown.

Among the genes that were highly expressed only at 3 dpi., IFN-ω1 (IFN-ω), DHX58 (ATP-dependent RNA helicase DHX58) and IFN-α (IFN-α) had 5.5- to 9-fold increases in expression with IFN-α showing the greatest up-regulation. IFNs are cytokines that induce antiviral responses in cells and play a major role in the defense against viral infection in vertebrates. There are two families of IFNs, type I and II, that have different gene sequences and functional properties. Mammalian type I IFNs constitute a multigene family with several subclasses among which the IFN-α and IFN- functional genes have been reported. In teleost fish, type I IFN-orthologous genes have been described and cloned from several species. Our results showed that IHNV, as expected, induces the high-level expression of the type I IFN genes, which in turn up-regulates the transcription of a number of immune genes. For the DETs that were found exclusively at 7 dpi., thirty showed fold changes ranging from -2.59 to 3.8 and had a KO term assigned. Among these, 19 were down-regulated (fold changes from -2.59 to -1.65). The KOs included K11275 (H1 5-histone H1/5), which is implicated in nucleosome assembly factors, and K00671 (NMT-glycylpeptide N-tetradecanoyltransferase), which is involved with enzymes such as acyltransferases. Eleven were up-regulated between 1.76- and 3.81-fold (Figure 4B). The APLNR apelin receptor gene (AR or APJ) or G-protein coupled receptor APJ is a class A (rhodopsin-like) receptor. It is involved in the regulation of cardiovascular function and implicated in pathologies such as diabetes and cancer but also stimulates angiogenesis in humans. Its precise role in viral infections has not been described, but as IHNV replicates in blood capillaries, it can be speculated that the up-regulation of this gene is a host reaction to the predictable tissue damage occurring at this advanced infection stage.

The SLCBA gene was another gene in this group. Many viruses have evolved mechanisms to alter mitochondrial function and can mediate changes in mitochondrial uptake of Ca^+; thus, the IHNV induced up-regulation of the SLCBA gene (a member of the solute carrier family 8) may be related to the altered Na^+/Ca^+ exchanger and the viral modification of this cellular function.

The FABP7 gene was also up-regulated more than 2-fold after 7 days of IHNV infection. In humans, FABP7 has been associated with cancer. In mammals, the intracellular fatty acid-binding proteins (FABPs) are abundantly expressed in almost all tissues. Proposed roles for FABPs in humans include the assimilation of dietary lipids in the intestine, the targeting of liver lipids to catabolic and anabolic pathways, the regulation of lipid storage and lipid-mediated gene expression in adipose tissue and macrophages, and the maintenance of phospholipid membranes in neural tissues (Storch Thumser, 2010). In fish, the FABP gene family has been chosen by a few researchers as a model to study the fate of duplicated genes following whole genome duplication, but its role in fish metabolism is less well known. However, as FABPs have been associated with a high number of diseases and have an important role as controllers of global metabolism, their relevance may be extrapolated to the pathological changes taking place in the kidney tissue after infection.

The TNFSF14 gene is a member of the tumor necrosis factor ligand superfamily (also known as LIGHT), which plays important roles as a proinflammatory chemokine and cytokine, as well as inducing cell death and apoptosis and enhancing T cell survival, thus explaining its up-regulation in advanced viral infections. This gene has been isolated and characterized in zebrafish (Tian *et. al.*, 2012). Another of the overexpressed genes was speG or diamine N-acetyltransferase, which is related to TNF during inflammation processes, and both showed similar levels of expression (2-fold increase) in the infected kidneys at 7 dpi..

Among the uniquely expressed genes at 7 dpi., three were detected, Lyz, CDD or AID and TAC1, showing an approximate 2.5-fold increase in expression. Lysozyme (Lyz) is an important part of the innate immune response of fish and is present in mucus, plasma, lymphoid organs and other body fluids of fish. The main function of this enzyme is its lytic activity against bacteria, but an increase in lysozyme activity in fish blood can also be induced by infections or invasion by foreign material (Ellis, 2001). Cytidine deaminase (AID) is an essential regulator of B cell diversification. In humans, CDD expression and activity is tightly regulated, and deregulation is associated with diseases including cancer (Chaudhuri, Evans, Kumar, DiMenna, 2014); moreover CDD expression is related to chronic inflammation, which may explain the observed up-regulation during the process of viral infection. Tachykinin 1 peptide (TAC1) performs multiple physiological functions; some of which are related to inflammation. Tachykinins have been isolated from nervous and/or gastrointestinal tissues of all classes of vertebrates studied. In fish, two tachykinin-like peptides have recently been isolated from the skin of zebrafish (Mi *et. al.*, 2010).

Additionally, the most highly expressed gene at the 7 dpi. time point was the RABL5 gene. The Rab-like protein 5 (RABL5) gene belongs to the Ras-like GTPase superfamily and plays an important role in intracellular membrane trafficking. It has been associated with trafficking in sensory cilia and flagella of some parasitic protozoa, but in fish studies, its functions are scarcely understood. It has recently been found (Hu, Deng, Sun, 2011) that the over-expression of a RABL5 homologue enhances host resistance against the intracellular invasion of E. tarda in red drum (*S. ocellatus*). They demonstrated that a fish Rab1 GTPase regulates intracellular bacterial infection and thus is likely to play a role in bacteria-induced host immune defense. In mammals, Rab 5 is known to be involved in hepatitis C virus and dengue virus infections. The potential functions of these genes in the host-viral interaction remain un-investigated in fish, but it can be speculated that the RABL5 over-expression reported in the present work may be a host reaction against an intracellular invasive microorganism at a time when tissue damage has occurred.

E. Functional annotation by Gene Ontology (GO)

1. *Transcripts that are present in both (3 and 7 dpi.) time point samples*

Among the modulated DETs, 16 out of 33 had one or more GO annotations, while in similarly ex-

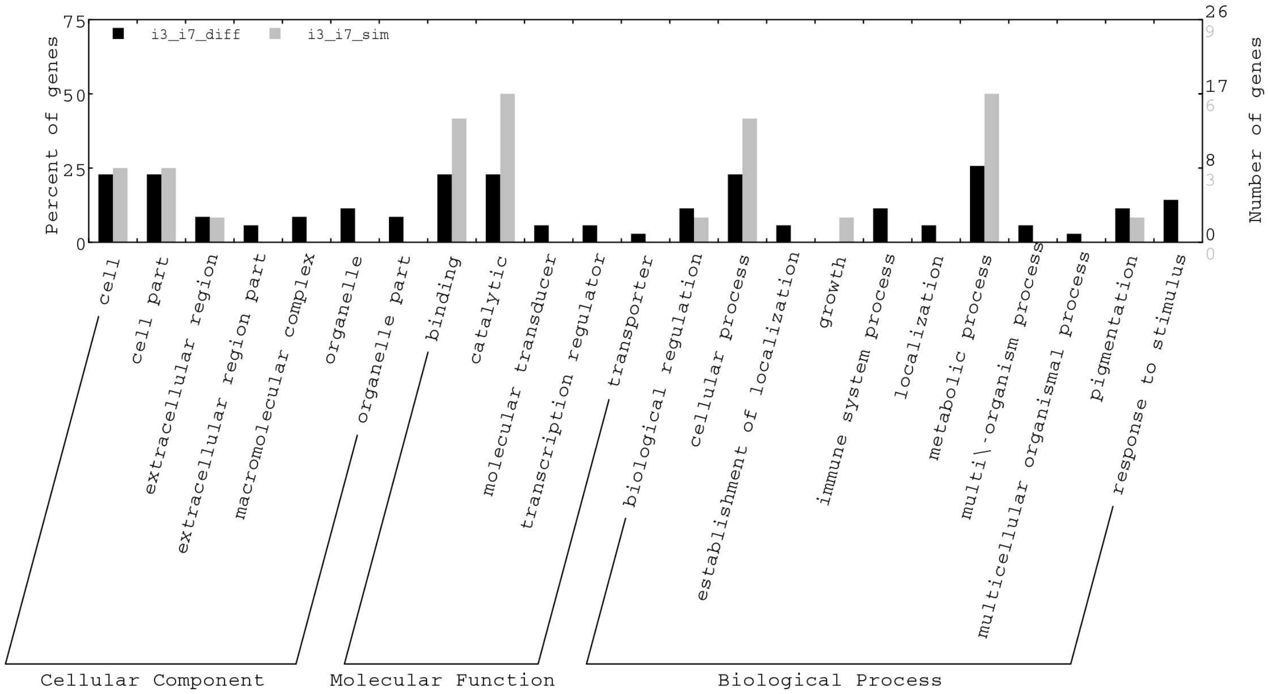

FIG. 5: Gene Ontology (GO) classification of differentially expressed transcripts (DETs) at 3 and 7 days post-IHNV infection (dpi.) (DETs that are present in both samples). GO assignment to these genes was based on high-score BLASTX matches to the *Danio rerio* proteins (TAIR-NCBI) using WEGO (Web Gene Ontology Annotation Plot) http://wego.genomics.org.cn/cgi-bin/wego/index.pl. The GO terms were processed and categorized under three main GO categories (biological process, molecular function, and cellular component); the left axis indicates the percentage of a specific category of genes in that main category (GO level=2). Differentially (A) and similarly (B) expressed transcripts (fold change) at 3 dpi./control vs. 7 dpi./control.

pressed DETs this number was 7 out of 14 (Table 3). DETs are involved in all three GO categories (biological, molecular and cellular), although biological processes and molecular functions are more represented than cellular components. Regarding molecular function, binding (GO:0005488) and catalytic activity (GO:0003824) were the most represented sub-categories at GO level 2 (Figure 5). These two sub-categories were associated with eight modulated DETs, while there were five similarly expressed DETs associated with the binding GO and five with the catalytic activity GO. The major subcategories for biological process were cellular processes (GO:0009987) and metabolic processes (GO:0008152). The cellular processes sub-category was associated with nine modulated DETs, and the metabolic processes, eight. Regarding similarly expressed DETs, six were associated with cellular processes and five with metabolic processes (Table 3). Immune system processes (GO:0002376) and response to stimulus (GO:0005576) were associated with four and five modulated genes, respectively, but with none of the similarly expressed genes. Regarding the IHNV infection time and the profiles of abundant genes recorded, at 3 dpi., higher numbers of genes were observed than those expressed at 7 dpi.. These DETs belong to all of the three categories, with those related to binding, cell parts, cellular and metabolic processes being the most numerous. The profiles of genes recorded at the 7 dpi. time point and distributed by category were quite different because many of the sub-categories did not show appreciable numbers of genes; exceptions were binding, catalytic activity, cellular and metabolic processes, where the number of recorded DETs were double those of the 3 dpi. time. This finding suggests a correlation between these processes and the more productive phase of viral infection when there are clinical symptoms and damage to multiple tissues.

2. *Transcripts that are present in a unique (3 or 7 dpi.) time point sample*

Regarding the genes that were identified exclusively at 3 or at 7 dpi., the percentages of annotated DETs associated with a GO term and the nature of these terms is shown in Table 3 and Figure 6. Approximately 50% of the DETs had one or more GO terms annotated, and the most represented categories and sub-categories were the same as those in Figure 6. As a general observation, the results suggest that, despite the fact that Gene Ontology characterizes the function of the gene products of differentially expressed genes during IHNV infection, it was not possible to discriminate among the different categories of DETs using this resource.

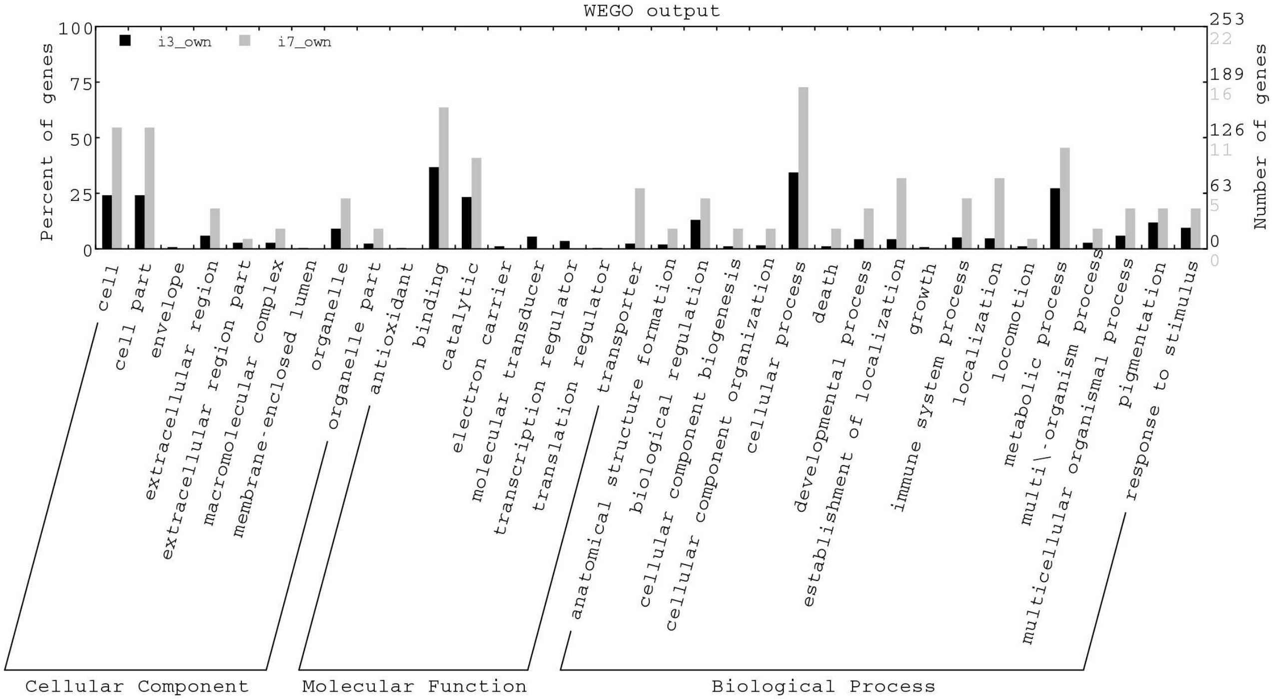

FIG. 6: Gene Ontology (GO) classification of differentially expressed transcripts (DETs) that were solely present in one of the fish groups (3 or 7 days post-IHNV infection (dpi.) samples). Gene Ontology (GO) assignment to these genes was based on high-score BLASTX matches to the *Danio rerio* proteins (TAIR-NCBI) using WEGO (Web Gene Ontology Annotation Plot) http://wego.genomics.org.cn/cgi-bin/wego/index.pl. The GO terms were classified into three main GO categories (biological process, molecular function, and cellular component); the left axis indicates the percentage of a specific category of genes in that main category (GO level=2). (A) DETs at 3 dpi. in relation to control fish and (B) DETs at 7 dpi. in relation to control fish.

F. Functional annotation by KO - Pathway analysis

KO annotation allows the generation of organism-specific pathways. To identify and increase the potential number of pathways, we have used the *Danio rerio* (dre) and the *Homo sapiens* (hsa) databases.

1. *Pathway analysis of transcripts that were present in both (3 and 7 dpi.) samples.*

There were a total of 58 pathways that contained modulated genes (genes with different expression levels) during the IHNV infection (see supporting information Table S3). The most representative pathways are shown in Figure 7A, and only the up-regulated genes are shown. The highest numbers of stimulated genes were those involved in amino acid metabolism, including alanine, aspartate and glutamate metabolism (map00250), taurine and hypotaurine metabolism (map00430), followed by the cytosolic DNA-sensing pathway (map04623), glyoxylate and dicarboxylate metabolism (map00630), which has been reported to be involved in controlling tight junctions and protein trafficking, and purine metabolism (map00230), which is involved in the inflammation process in murine models (Weissmuller, Eltzschig, Colgan, 2005). A lower percentage of genes were represented in other pathways that are of interest for understand the viral activity of pathways related to immunity and inflammatory responses, such as TPR channels (map04750), peroxisomes (map04146), NK cell-mediated cytotoxicity (map04650) and the MAPK signaling pathway (map04010). Viral proteins have an enormous ability to interact with host proteins, altering cellular physiology and exploiting host-cell pathways (Hilchie, Wuerth, Hancock, 2013). Therefore, IHNV infections in rainbow trout have been associated with histopathological damage that first manifests as necrosis (Yasutake Amend, 1972). Moreover, the release of cellular contents during necrosis is highly inflammatory and induces changes in gene expression that regulate TRP channels (map04750) at both 3 and 7 dpi.. In general, most of the up-regulated genes were observed in this cluster. However, NF-kappa B (map04064) signal transduction experiences a range of regulation from moderate to severe in the head kidneys of infected trout at 3 and 7 dpi.. NF-kappa B is the generic name for a family of transcription factors that function as dimers and regulate the genes involved in immunity, inflammation and cell survival. NF-kappa B is a ubiquitous transcription factor, and its properties seem to be most ex-

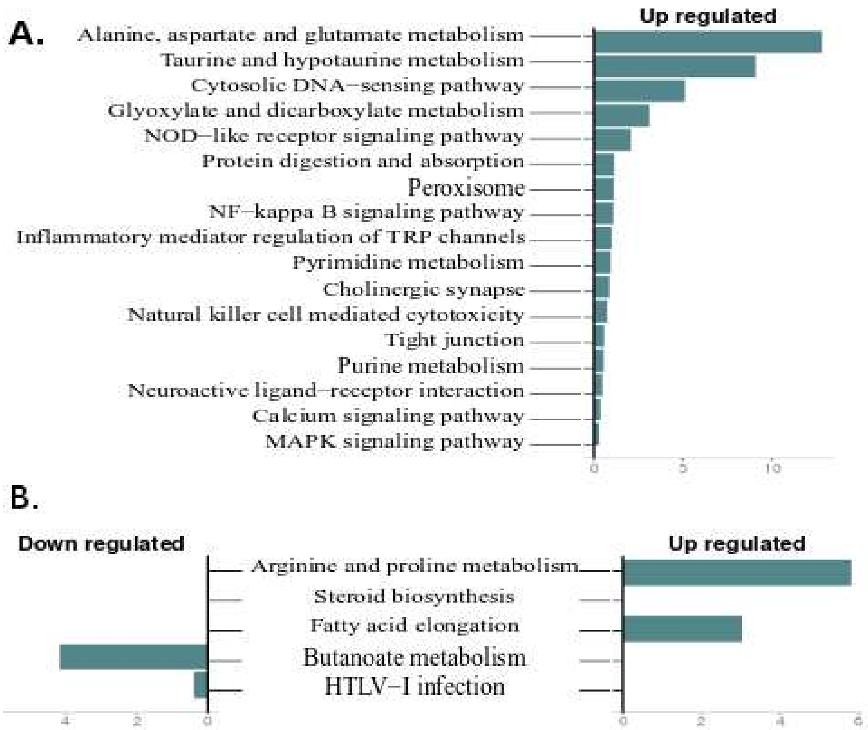

FIG. 7: Represented pathways from orthologous genes of differentially expressed transcripts (DETs) at 3 and 7 days post-IHNV infection (dpi.) (DETs that are present in both samples). Most of the transcripts shown were at least two-fold differentially regulated and statistically significant ($p<0.05$) in pairwise T-tests compared with control fish. The x-axis of the histogram represents the percentage of DETs from *O. mykiss* over the total number of genes involved in each pathway from the reference orthologous species *Danio rerio* (dre) or *Homo sapiens* (hsa) in the KEGG database. On the left are down-regulated DETs relative to the nave fish (control), and on the right are up-regulated DETs. Differentially expressed fold change transcripts from 3 to 7 dpi. (A): (3 dpi./control vs. 7 dpi./control), and similarly expressed fold change transcripts from 3 to 7 dpi. (B): (3 dpi./control vs. 7 dpi./control)).

tensively exploited in the cells of the immune system. It regulates a large number of genes, including many involved in bacterial and viral infections. The post-translational activation of NF-kappa B in response to many pathological signals is rapid; it directly participates in cytoplasmic/nuclear signaling, and it potently activates the transcription of a great variety of genes encoding immunologically relevant proteins (Baeuerle Henkel, 1994). The activation of the NF-kappa B pro-inflammatory pathway by the IHNV was first reported in 2007 by Miller *et. al.* (Miller *et. al.*, 2007). There are several pathways leading to NF-kappa B activation. In respect to the set of genes with similar expression levels at 3 and 7 dpi., there were a total of 32 pathways in the head kidney, for which most of the genes were up-regulated (see supporting information Table S3). The most enriched pathways (Figure 7B) were those related to metabolism, such as arginine and proline metabolism (map00330), steroid biosynthesis (map00100), and fatty acid elongation (map01212). This up-regulation suggested an increase in fatty acid synthesis of cell membrane phospholipids such as arachidonic acid, which can be converted by various enzymes into eicosanoid signaling molecules, exerting complex control over inflammation or immunity (Holen, Lie, Araujo, Olsvik, 2012) and HTLV-1 infection (map05166).

2. Pathway analysis of transcripts that are present in a unique (3 or 7 dpi.) sample

Most of the immunological pathways were found to be activated at 3 dpi. (170 KO). Those having a higher percentage of genes are represented in Figure 8A with most having up-regulated genes induced by IHNV. Here, we found viral innate recognition pathways, including the retinoic acid inducible gene I (RIG-I) and TLR. These genes have previously been reported in studies of targeted viral evasion (Kawai and Akira, 2006). Otherwise, in our results, most of the pathways implicated at 3 dpi. had up-regulated KO ($\sim$80%). The regulation of the autophagy pathway (map04140) was the most gene-enriched pathway (as approximately 30% of the gene KOs analyzed were involved in this pathway) followed by the cytosolic DNA-sensing pathway (map04623). Autophagy maintains cell, tissue and organism homeostasis through degradation, and additional functions have recently been reported, such as the regulation of metabolism,

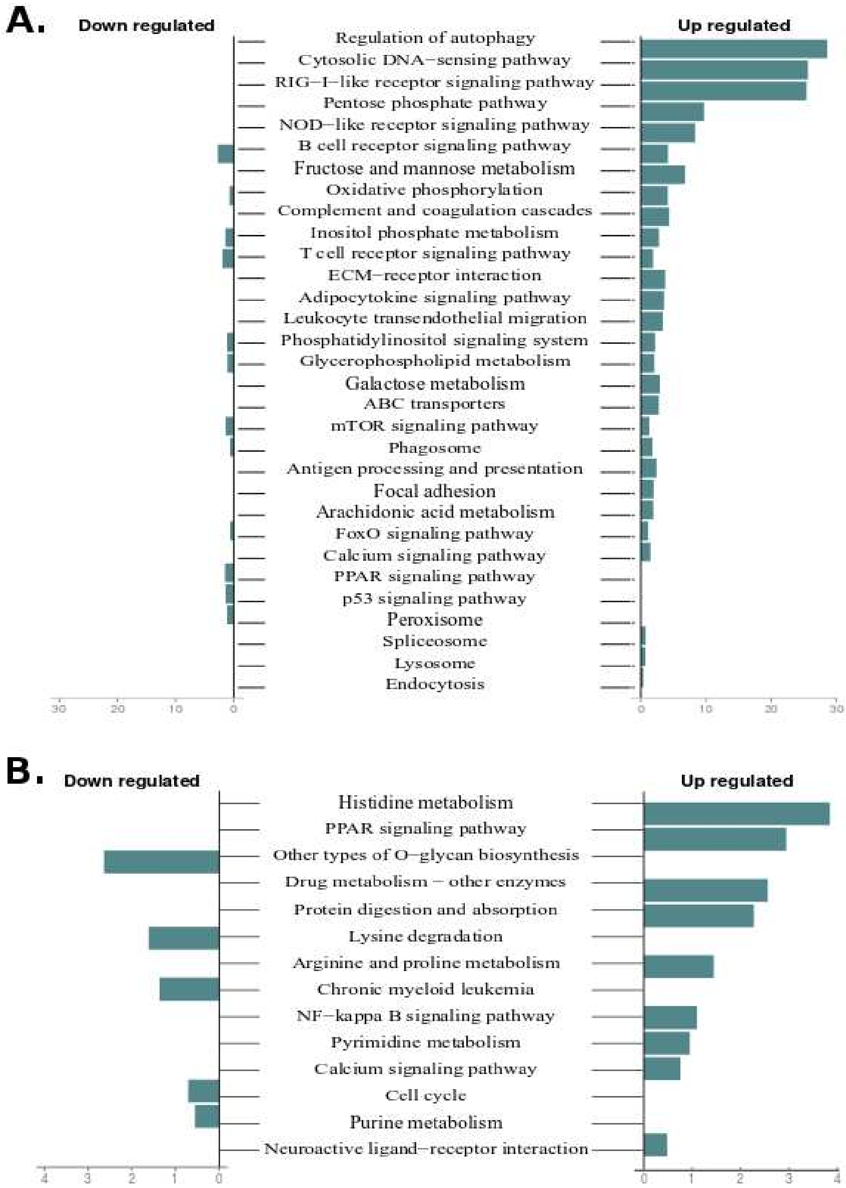

FIG. 8: Represented pathways from orthologous genes that were exclusively present in one of the post-IHNV infection samples (3 or 7 dpi.). (A) DETs at 3 dpi. relative to control fish and (B) DETs at 7 dpi. relative to control fish. Most of the transcripts shown were at least two-fold differentially and significantly regulated ($p<0.05$) in pairwise T-test compared with control fish. The x-axis of the histogram represents the percentage of DETs from *O. mykiss* head kidney over the total number of genes involved in each pathway. The references used were from orthologous species (*Danio rerio* (dre) or *Homo sapiens* (hsa)) in the KEGG database.

membrane transport and modulation of host defenses. This result is understandable given the high participation of genes in such a pathway when examining virally affected tissues.

At 7 dpi., 34 pathways were involved, and the most representative are shown in Figure 8B. Histidine metabolism (map00340) and PPAR signaling pathways (map03320) had the highest percentage of upregulated target genes followed by drug metabolism (map00983) and protein digestion and absorption (map04974). All of these pathways are relevant for viral replication as this can trigger an effective IFN antiviral response; host pattern recognition receptors have to detect pathogen associated molecular patterns derived from the invading virus and initiate different adaptor-dependent signaling cascades (Feng *et. al.*, 2015). Symptomatic infection exhibits a distinct molecular signature over time, where interactions occur in a complicated and diverse manner between the components of the host immune system. Some experimental analyses have elucidated the mechanistic activation and modulation of the host response to

IHNV infection. However, the knowledge of massive transcriptome responses focusing on the immune-related responses to IHNV infection are still preliminary. Our present results indicated that at 3 and 7 dpi., many immune-related genes were identified as up-regulated. We can divide the analyzed genes into two gene-type clusters: one in which genes whose temporal expression patterns differentiate immune responses of asymptomatic from symptomatic fish, and the other in which clusters represent the molecular signatures of unique and contrasting temporal dynamics. However, to deeply understand the factors affecting the expression of these types of gene clusters requires further work. Some of the pathways in which immune genes were involved are described next.

G. Immunological pathways

To identify the significance of genes implicated in the immune pathways that are active in asymptomatic and symptomatic IHNV infection in rainbow trout, the 6875 and 5857 DETs at 3 and 7 dpi., respectively, were mapped to the signaling pathways found in KEGG. The immune pathways in rainbow trout containing the genes involved at 3 dpi. and 7 dpi. are shown in Figure 9A and 9B, respectively. Most of the genes implicated were up-regulated at both 3 and 7 dpi., with 3 dpi. having more enriched genes compared to 7 dpi.. This may be explained by the IHNV infective course that would induce mechanisms to evade or limit innate host defenses, disrupt normal cellular processes, and gain preferential transcription and translation of viral genes. The main pathway among those involved in IHNV immunological processes were the Toll-like receptor signaling pathway (dre04620), followed by the JAK-STAT signaling pathway (dre04630), cytokine-cytokine receptor interaction (dre04060), the TNF signaling pathway (hsa04668), hematopoietic cell lineage (hsa04640), the chemokine signaling pathway (hsa04062), apoptosis (dre04210) and natural killer cell-mediated cytotoxicity (hsa04650). These contained more up-regulated than down-regulated genes. Moreover, some of those pathways are presented as supporting information in Figures S1-S5, including cytokine-cytokine receptor interaction (dre04060) and those involved in the growth and differentiation of immune response cells, such as the hematopoietic cell lineage (hsa04640), the TNF signaling pathway (hsa04668), the chemokine signaling pathway (hsa04062) and finally, natural killer cell-mediated cytotoxicity (hsa04650), at both 3 and 7 dpi..

In the present work, detailed gene expression after IHNV infection has been illustrated in some immune pathways, such as TLRs (one of the first immunological pathways involved in a viral infection), JAK-STAT (one of the most reported and representative immunological pathways in fishes) and apoptosis (a pathway with strong influence on host cells that can explain some symptoms during the course of IHNV infection).

H. Toll-like receptor signaling pathway (map04620):

Toll-like receptors (TLRs) are the best understood of the innate immune receptors that detect pattern recognition receptor (PRR) signaling for pathogen-associated molecular patterns (PAMPs). The fish TLRs and the factors involved in their signaling cascade have high structural similarity to the mammalian TLR system. However, the fish TLRs also exhibit very distinct features and increased diversity, which is likely derived from their diverse evolutionary history and the distinct environments that they occupy. Six non-mammalian TLRs have been identified in fish. TLRs are the key transducers of the type-I IFN response, mediated by the activation of interferon-regulatory factors (IRF-family members). They are activated by MyD88 (myeloid differentiation primary-response protein 88) and have been described in zebrafish (Meijer *et. al.*, 2004). TLR activation can also up-regulate co-stimulatory molecules that are important for T cell clonal expansion and the secretion of immunomodulatory cytokines to direct T cell differentiation into effectors (Hemmi *et. al.*, 2000). TLRs are able to regulate neutrophil migration, activation, and apoptosis and B-cell activation and survival (Kondo *et. al.*, 1997). TLRs are also an important link between innate and adaptive immunity through their presence in dendritic cells (DCs) (Takahashi, 2003). In fish, their activity is now being increasingly studied (Pietretti Wiegertjes, 2014).

The results obtained here (Figure 10A) show the up-regulation of most of the genes involved in this pathway, including TLR1, TLR3, TLR5, and MyD88, which were significantly up-regulated at both 3 and 7 dpi., except for TLR1 at 7 dpi.. TLR7 and TLR8, which are localized in the endosome, were also up-regulated. It is known that mammalian TLRs 7 and 8 are responsible for recognizing viral single-stranded RNA (ssRNA) and are activated by synthetic antiviral imidazoquinoline compounds (Taro Kawai and Akira, 2007). Up-regulation similar to that reported here was also reported in salmon after IHNV infection by Miller *et. al.* (2007) (Miller *et. al.*, 2007), who suggested its connection to the recognition of ssRNA in cellular sites where the virus was replicating. Other studies in turbot infected with VHSV or challenged after vaccination analyzed transcriptional changes by microarrays. This study also reported the modulation of six types of TLRs, one of them being TLR 8 (Pereiro *et. al.*, 2014).

With regard to the TLR pathway, our results indicate that many of the involved genes were up-regulated at both 3 and 7 dpi.. For example, TLR3 is directly connected to MyD88 activation and the pathway that modulates IRF-7 and ultimately IFN-α. TLR3 has spurred research to determine its function and potential as a good adjuvant candidate for vaccines against viral diseases relevant to the farming industry (Rodriguez, Wiens, Purcell, Palti, 2005).

Other studies have recently described the up-

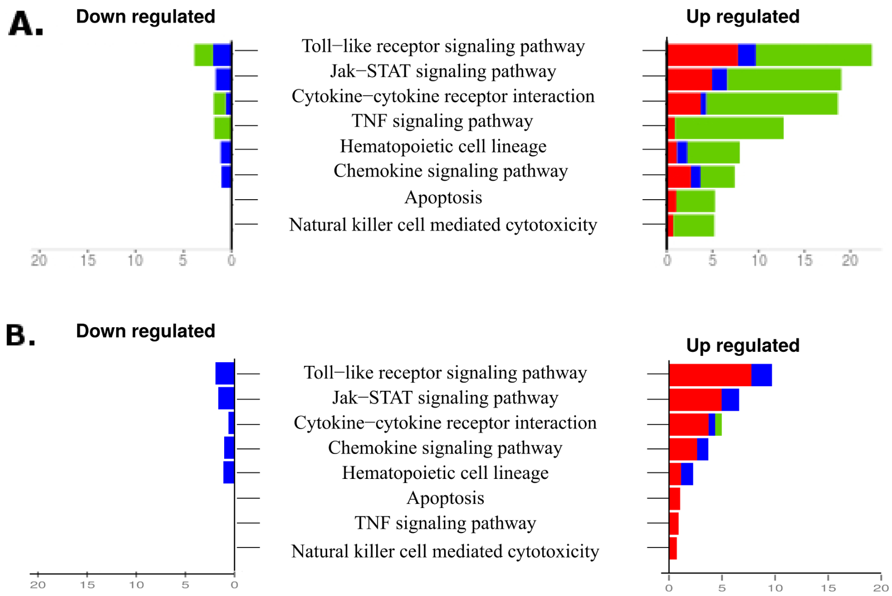

FIG. 9: Percentage of differentially expressed transcripts (DETs) relative to control fish, involved in main immune pathways in the head kidneys of trout following IHNV infection at 3 (A) and 7 (B) days post-infection (dpi.) relative to control fish. Percentage of DETs (fold change) expressed at both 3 and 7 dpi.: (red) differently; (blue) similarly and (green) DETs that are present in just one sample.

regulation of TLR3 after infection with infectious anemia salmon disease, which suggests a role for TLR3 in the antiviral immune response (Arnemo, Kavaliauskis, Gjen, 2014). The hallmark of endosomal TLRs 3, 7, 8 and 9, which sense nucleic acids, is the induction of type I IFNs (Schroder, Sweet, Hume, 2006). Thus, all of these findings correlate with the expected activation of IFN genes that follows viral infection.

Regarding our results illustrated in the TLR pathway, some other genes were up-regulated exclusively at 3 dpi., such as TLR1 and Casp8, which are related to apoptosis, IRAK1, and the pro-inflammatory IL6 and IL12 genes. Among the genes down-regulated at 3 dpi., cathepsin, MKK3 and MKK4 are highlighted in Figure 10A. In humans, the protein encoded by the cathepsin K (CTSK) gene is a lysosomal cysteine protease involved in bone remodeling and resorption. The enzyme's ability to catabolize elastin, collagen, and gelatin allows it to break down bone and cartilage. The MKK3 gene is a specific activator of p38 MAP kinase that is independent of the JNK and ERK signaling pathways. A second MAP kinase kinase, MKK4, phosphorylates and activates both p38 MAP kinase and JNK in vitro. Some studies have implicated p38 MAP kinase in the phosphorylation of the small heat shock protein Hsp27, in increased cytokine expression and in programmed cell death (Raingeaud, Whitmarsh, Barrett, Drijard, Davis, 1996). These are enzymes related to inflammation and the response to stress, and it can be speculated that their early down-regulation may be mediated by the virus to avoid macrophage trafficking to damaged cells. As a general observation, higher numbers of genes were up-regulated at 3 dpi. probably because all of the machinery of the invaded cells is supporting maximum transcriptional activation, together with the responses modulated by the virus to survive in the host. However, the profusion of these biological events is not reflected in apparent disease symptoms, as this happens later at 7 dpi..

It is well known that viruses employ multiple strategies to evade host signaling mediated by TLR and RIG-I-like receptor (RLR). Some viral proteins can directly interact with host signaling molecules to inhibit their function. As a key transcription factor, IRF3 is a frequent target of viral proteins that inhibit its phosphorylation because phosphorylated IRF3 forms homodimers and translocates to the nucleus where it induces IFNB and other relevant genes, such as the gene encoding the chemokine CXC-chemokine ligand 10 (CXCL10) (Honda Taniguchi, 2006). In the brains of mice infected with rabies virus (RABV), also a rhabdovirus, it has been shown that the enhancement of blood-brain barrier (BBB) permeability is modulated by the expression of CXCL10 and a reduction in tight junctions (TJ) (Chai, She, Huang, Fu, 2015). These processes may help to explain our results showing gene expression modulation at initial infection times in comparison to symptomatic viral infections. In contrast, some genes were down-regulated at 3 dpi. but up-regulated at 7 dpi., including AP-1 and PI3K, which are involved in many pathways, such as TLR, JAK-STAT and apoptosis. AP-1 controls a number of cellular processes, including differentiation, proliferation, and apoptosis. AP-1 activity is induced by growth factors, cytokines, neurotransmitters, polypeptide hormones, cell-matrix interactions, bacterial and viral infections, and a variety of

A.

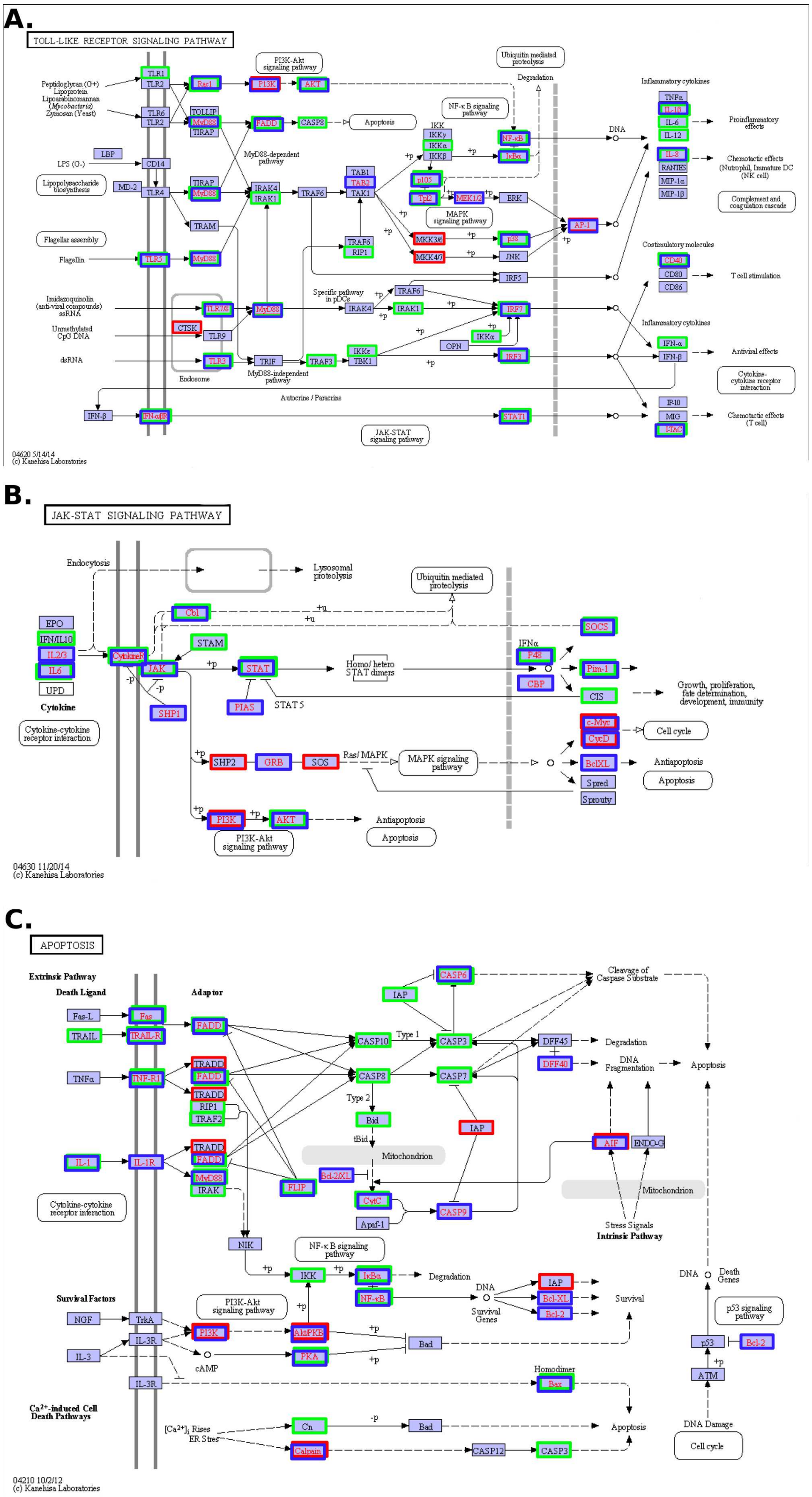

FIG. 10: Significant enrichment of immune pathway DETs in the head kidneys of rainbow trout during IHNV infection relative to control fish. A p-value of <0.05 was selected as the significance cutoff. (A) The toll-like signaling pathway, (B) the JAK-STAT signaling pathway, (C) apoptosis. The gene boxes containing the orthologous rainbow trout genes and their relationships were obtained from the corresponding zebrafish or human KEGG pathways. Up-regulation (green) and the down-regulation (red) of DETs during IHNV infection at 3 dpi. vs. the control group. The up-regulation of DETs during IHNV infection at 7 dpi. relative to the control fish (blue).

physical and chemical stresses. These stimuli activate mitogen-activated protein kinase (MAPK) cascades that enhance AP-1 activity through the phosphorylation of distinct substrates. AP-1 transcription factors are involved in both the induction and prevention of apoptosis, and the exact outcome is highly tissue and developmental-stage-specific (Shaulian Karin, 2002). Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase and a second messenger essential for the translocation of Akt to the plasma membrane. The relevance of the modulation of these genes is explained below in the JAK-STAT pathway. The down-regulation of these genes would favor viral replication in cells at early time points, but the situation changed after 7 dpi., when the host has developed some potential (cytokines, ILs, cellular immunity) to take the control of the expression of genes initially modulated by the invading virus. Alternatively, it can be speculated that the virus is able to modulate some genes involved in the innate immune response, trying to evade early host defense; later, once the cascade of reactions has begun, the inhibition of viral replication will depend on the extent of the antiviral state achieved.

I. JAK-STAT signaling pathway (map04630)

Immunity to infection is achieved by a wide array of complex and interconnected pathways. Initially, viruses that enter an organism induce genes in the context of interferon signaling through the conserved JAK-STAT pathway and up-regulate the expression of a high number of IFN-stimulated genes. An antiviral state is then established, and the transcription of antiviral genes is promoted through a single pathway. This pathway involves the Janus kinase/signal transducers (Jak kinases) and the activators of transcription factors belonging to the STAT family. The JAK-STAT pathway is one of a handful of pleiotropic cascades used to transduce a multitude of signals for development and homeostasis in animals.

According to our results (Figure 10B), at 3 dpi., some genes, such as SOS, PI3K, SHP2, c-Myc and CycD, which are involved in the cell cycle and MAPK signaling pathways, were down-regulated; this would suggest a viral mechanism that interferes with normal cell processes and the first host responses to the infection. However, there is no compelling evidence that IHNV has a direct mechanism to antagonize IFN, with the exception of the host cell shut-off phenomenon. In a recent work, Encinas *et. al.* (2013) described the regulation of genes in zebrafish infected with another rhabdovirus, spring viremia carp virus (SVCV), and also observed down-regulation in several pathways and shutoff effects at early stages of infection, probably favoring viral replication. Viral shutoff may result in the failure to induce immune responses, but genes with IFN response elements in their promoters have the ability to resist host-cell shutoff. However, rhabdoviruses such as IHNV are sensitive to the effects of IFN once induced (Stertz *et. al.*, 2007) and, despite this initial down-regulation of some genes, many more were up-regulated by the virus at 3 and 7 dpi., such as AKT, JAK, and STAT, among others. These genes participate in anti-apoptotic processes and are essential for IFN production. In mammals, the JAK-STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors. Following the binding of cytokines to their cognate receptor, STATs are activated by members of the JAK family of tyrosine kinases; the STATs are recruited to the receptor bound JAKs either directly or by SH2 domain-containing adapter proteins that have high affinities for phosphorylated tyrosine residues in proteins and are activated by members of the JAK family of tyrosine kinases. Once STATs are activated, JAKs mediate the recruitment of molecules, such as the MAP kinases, PI3 kinase and others. These molecules process downstream signals via the Ras-Raf-MAP kinase and PI3 kinase pathways, which results in the activation of additional transcription factors (Schabbauer *et. al.*, 2008).

As described above in the TLR pathway, our results showed that PI3K was down-regulated at 3 dpi. and up-regulated later, at 7 dpi., and this late up-regulation was also observed for AKT. This finding suggests the initial host activation of the PI3K/AKT pathway followed by a viral action to control programmed cell death to create an opportunity to replicate before the cells lyse. The PI3K/AKT pathway is an intracellular signaling pathway important in regulating the cell cycle. PI3K activation phosphorylates and activates AKT, localizing it in the plasma membrane. Once active, Akt can control key cellular processes by phosphorylating substrates involved in apoptosis, protein synthesis, metabolism, and cell cycle control. In mammals, this pathway is overactive in cancer to induce apoptosis.

Other down-regulated genes at 3 dpi. were Shp2 and SOS. Shp2 is a ubiquitous protein tyrosine phosphatase (PTP), which plays important biological roles in response to various growth factors, hormones and cytokines. Numerous evidence indicates that it is one of the rare PTPs that promotes the activation, rather than the down-regulation, of intracellular signaling pathways, such as Ras/MAPK. The SOS gene induces a global response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis are induced. The activation of SOS genes occurs after DNA damage by the accumulation of single stranded DNA (ssDNA) regions (Dance, Montagner, Salles, Yart, Raynal, 2008).

The fish in the present work had phenotypic changes as a consequence of disease progression. The results of pathway enrichment here made evident some differences in the up- or down-regulation of several groups of genes at 3 or 7 dpi.. These differences are highlighted below.

J. Apoptosis (map04210)

Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis and is critical for the development and maintenance of healthy tissues. The two major pathways of apoptosis are the extrinsic (Fas and other TNFR superfamily members and ligands) and the intrinsic (mitochondria-associated) pathways, both of which are found in the cytoplasm. The extrinsic pathway is triggered by death receptor engagement, which initiates a signaling cascade mediated by caspase-8 activation. Caspase-8 both feeds directly into caspase-3 activation and stimulates the release of cytochrome c by the mitochondria (Colell *et. al.*, 2007). The activation of caspases is a marker for cellular damage in disease. Our results (Figure 10C) show that caspase-8 and caspase-3, among other caspases, were up-regulated by IHNV at 3 dpi.; therefore, caspase-3 activation prevents the degradation of cellular proteins necessary to maintain cell survival and integrity. However, at 3 dpi., the up-regulation of inhibitor of apoptosis (IAP) genes was also detected. IAPs are involved in suppressing the host-cell death response to viral infection. Additionally, at both 3 and 7 dpi., we found that most of the genes related to apoptosis were up-regulated. Altogether, our results illustrate numerous key apoptosis-signaling genes in the highlighted pathway. The infected cells induce apoptosis as a defense mechanism to interfere with viral replication and dispersion at early times post-infection, but after 7 dpi., with evident disease, the apoptotic events may also be a consequence of virus spread and tissue damage.

The extensive induction of caspase genes and other genes related to apoptosis was also described by Pereiro *et. al.* (2014) in a microarray study of turbot infected with VHSV, another relevant rhabdovirus affecting fish (Pereiro et al., 2014). However, in their study, the transcriptional profiles of vaccinated and challenged fish were quite different, as the existence of a specific response and a reduction in viral transcription avoid the activation of apoptotic mechanisms. The down-regulation of protein synthesis (shutoff) is another effect of IHNV infection in the host cell. In infection-associated apoptosis, there is the question of whether the cell death is due to viral factors or to the host antiviral response. Both factors may be responsible. For instance, in mammals, reverse genetic analysis of the rhabdovirus vesicular stomatitis virus (VSV) revealed that apoptosis occurs by two distinct pathways: one is associated with the matrix (M) protein and the other relies on host gene expression (Kopecky, Willingham, Lyles, 2001). IHNV M protein-mediated apoptosis may be a consequence of the host-cell shutoff activity because the suppression of host cellular gene expression and translation is most likely incompatible with long-term cell survival; it has been reported that the NV protein of IHNV acts in delaying apoptosis and suppressing the host IFN system to prolong viral replication (Ammayappan Vakharia, 2011). IHNV replication characteristically leads to cytopathic effects (CPEs) in cells. CPEs may be due to the viral disruption of cytoskeletal elements or host-induced apoptosis (Lyles, 2000). Similar to VSV, the IHNV M protein directly induces morphological changes consistent with apoptosis in cell culture. In a study conducted in cell cultures, Chiou *et. al.* (2000) demonstrated that the expression of IHNV M protein alone may be responsible for the shutdown and for the induction of programmed cell death; they suggested a significant role of programmed cell death in IHNV infection in fish (Chiou, Kim, Ormonde, Leong, 2000). Our data showed a very coordinated immune response after IHNV infection in a hematopoietic organ, the head kidney; however, the positive control of apoptosis seems to occur, which agrees with previous observations by Miller *et. al.* (2007) (Miller *et. al.*, 2007); they point to the viral strategy of blocking the intracellular recognition systems of defense early in infection.

Some of the genes in the apoptosis pathway, such as calpain, IAP, AIF and FADD, were down-regulated at 3 dpi. but up-regulated at 7 dpi., and differences in expression may be relevant to understanding the progression of viral infection.

Calpains are a family of cytosolic cysteine proteinases that have been implicated, among other biological processes, in apoptotic cell death and appear to be an essential component of necrosis (Khorchid Ikura, 2002). IAP (aryl hydrocarbon receptor interacting protein) can regulate the expression of many xenobiotic metabolizing enzymes. Germline mutations in the AIP gene predispose humans to the development of pituitary adenomas (Helivaara *et. al.*, 2009), and AIP is modulated by the immunophilin homolog hepatitis B virus X-associated protein 2 (Petrulis, Hord, Perdew, 2000). AIF (apoptosis inducing factor) is a flavoprotein involved in initiating a caspase-independent pathway of apoptosis (positive intrinsic regulator of apoptosis) by causing DNA fragmentation and chromatin condensation (Cand *et. al.*, n.d.) to induce programmed cell death. Fas-associated protein with death domain (FADD), also called MORT1, is an adaptor protein that bridges members of the tumor necrosis factor receptor superfamily, such as the Fas-receptor, to procaspases 8 and 10 to form the death-inducing signaling complex (DISC) during apoptosis. In addition to its most well-known role in apoptosis, FADD has also been shown to play a role in other processes, including proliferation, cell cycle regulation and development.

The tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) is an adaptor protein that was down-regulated at 3 dpi.. It is a death domain-containing adaptor molecule that interacts with TNFRSF1A/TNFR1 and mediates programmed cell death signaling and NF-kappa B activation. This protein binds the adaptor protein TRAF2, reduces the recruitment of inhibitor-of-apoptosis proteins (IAPs) by TRAF2, and thus suppresses TRAF2-mediated apoptosis. This protein can also interact with receptor TNFRSF6/FAS and adaptor protein FADD/MORT1 and is involved in the Fas-induced cell death pathway. From our results, it can be deduced that apoptosis, which is an important event induced by the host to eliminate virus-invaded cells, is inhibited at 3 dpi., suggesting a viral action to evade immune responses. Alternatively, it is possible that 3 dpi. is an early time of infection and this mechanism of defense is not yet stimulated. The genes were up-regulated later, at 7 dpi., which indicates that the apoptosis process proceeds, resulting in the loss of cell and functional mechanisms in the host. These events may explain the beginning of symptoms and signaling at 7 dpi..

K. Other immunological pathways:

With respect to the pathways included in the supporting information (Figures S1-S5), the chemokine signaling pathway (map04062) contains genes for chemo-attractant peptides that provide directional cues for cell trafficking and thus are vital for the protective host response; also, chemokines regulate abundant biological processes of hematopoietic cells leading to cellular activation, differentiation and survival. Our results show that SOS and Rho-associated kinase (Rho-kinase/ROCK/ROK) genes were down-regulated at 3 dpi.. ROCK is an effector of the small GTPase Rho and belongs to the AGC family of kinases. Rho-kinase has pleiotropic functions, including the regulation of cellular contraction, motility, morphology, polarity, cell division, and gene expression. Pharmacological analyses have revealed that Rho-kinase is involved in a wide range of diseases, such as vasospasm, pulmonary hypertension, nerve injury, and glaucoma, and is therefore considered to be a potential therapeutic target in mammals. Tumor necrosis factor (TNF) is involved in a wide range of intracellular signaling pathways, including apoptosis and cell survival as well as inflammation and immunity. Activated TNF requires the trimerization of TNFR1 or TNFR2. TNFR1 is expressed by nearly all cells and is the major receptor for TNF (also called TNF-α). In contrast, TNFR2 is expressed in limited cells, such as CD4 and CD8 T lymphocytes, endothelial cells, and others in mammals. It is the receptor for both TNF and LTA (also called TNF-β). Here, our results from 3 and 7 dpi. showed that TNFR1, TNFR2 and many other genes were up-regulated; thus, TNFR1 signaling induces the activation of many genes that are primarily controlled by two distinct pathways: the NF-kappa B pathway/MAPK cascade and apoptosis/necrosis. TNFR2 signaling can be activated by the PI3K-dependent NF-kappa B pathway and JNK pathway leading to survival. This cascade of events is a result observed in other pathways described in the present work. Since the first reports from Purcell *et. al.* (2010) (Purcell, Lapatra, Woodson, Kurath, Winton, 2010) on the modulation of rainbow trout gene expression in IHNV infections, several studies have focused on the transcriptional profiles of this and other fish viruses; most of them have highlighted immune responses. However, there are yet few descriptions of the pathways following IHNV infection and their interconnections are still poorly understood. The relevance of the NF-kappa B pathway, among others, in the IHNV infection was previously reported by Miller *et. al.* in Atlantic salmon using microarray technology to follow the up-regulation of defense pathways in response to IHNV infection (Miller *et. al.*, 2007).

The activation of the I-kappa B kinase/NF-kappa B cascade has also been described in turbot that received a DNA vaccine and later challenge with the rhabdovirus viral hemorrhagic septicemia virus (VHSV) (Pereiro *et. al.*, 2014). Moreover, this work describes the regulation of a number of processes directly or indirectly related to immune responses, as well as to apoptosis and proteolysis. Their results, as well as those described in the current work, are consistent with the foreseeable changes that occur in an infected host. After a viral infection, the up-regulation of immune genes is rapid, as would be expected. Teleost fish possess the principal components of innate and adaptive immunity found in other vertebrates. The survival of acute rhabdoviral infection is also dependent on innate immunity, particularly the interferon (IFN) system that is induced in response to infection. Purcell *et. al.* (2012) reported that, despite the fact that rhabdoviruses are sensitive to the effects of IFN, its induction is not always sufficient to prevent viral replication. Indeed, the most virulent strains can continue to replicate due to the abilities of the M protein to mediate host-cell shutoff. The non-virion (NV) protein, which acts through programmed cell death and suppresses functional IFN, may also be obviated (Purcell, Laing, Winton, 2012).

L. Validation of DETs using qPCR

Seven genes associated with the immune response and covering the full range of expression changes observed were selected and analyzed by q-PCR (to validate the results of the RNA-seq analysis). Most of the results were consistent with those of the RNA-seq analysis (Figure 11A and 11B). The transcript expression levels obtained by these two methods were highly correlated with a significant coefficient of determination of 0.89 (p-value <0.01). The relative expression levels of the viral G gene were also determined as an indirect method of evaluating the viral load at 3 and 7 dpi. in the target organ (Figure 11C). The highest expression level was observed at 3 dpi. in comparison with 7 dpi., indicating that viral replication peaked at early times. After 7 days, the tissue damage is surely extensive, and the viral replication is restricted to intact cells. These results may explain the higher expression levels of immune genes and general responsiveness at 3 dpi..

IV. CONCLUSIONS:

In summary, using next generation sequencing technology, a total of 6875 DETs and 5875 DETs were sig-

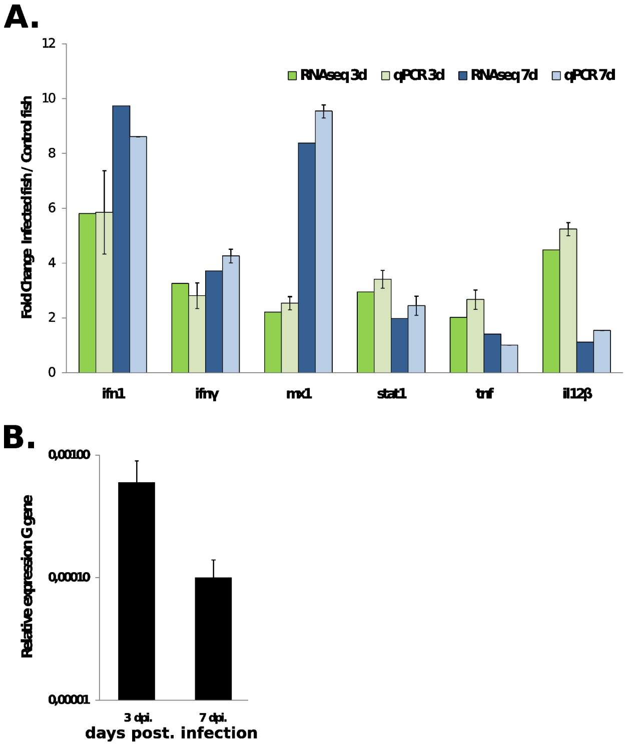

FIG. 11: Validation of the RNA-seq data by quantitative RT-PCR. Seven of the genes involved in immunity that were significant differentially regulated in the RNA-seq study were selected to validate these findings by RT-qPCR. Fold changes (log2 ratios) in genes at 3 days post-IHNV infection (dpi.) relative to the control fish (A), and (B) fold changes in genes at 7 dpi. relative to control fish. The transcript expression levels of the selected genes were each normalized to that of the endogenous control EF-1 gene (Ct method). (C). The relative quantitation of the G gene from IHNV was not shown in the RNA-seq study, but it is an important gene that reflects the host IHNV viral load; it may have an influence on the gene expression levels at 3 and 7 dpi.. The expression of the G gene can be related to the gene expression at 3 and 7 days post-infection. The relative quantitation of the G gene from IHNV virus was calculated using the Ct method, with the endogenous control gene (actin) as the normalizing gene following RT-PCR.

nificantly altered during asymptomatic (3 dpi.) and symptomatic (7 dpi.) IHNV infection, respectively, and 3141 of these transcripts were common to the fish analyzed at both time points in the head kidneys of rainbow trout. Only 2785 were down-regulated between -3.6819- and -0.0484-fold, while the remaining 4090 were up-regulated from 0.07- to 9.75-fold in the 3 dpi. sample. Additionally, 2247 DETs were down-regulated by -4.13- to -0.079-fold, and the remaining 3610 transcripts were up-regulated by 0.049- to 6.08-fold at 7 dpi.. There were 198 DETs expressed uniquely at 3 dpi., most of them up-regulated from 1.66- to 9.31-fold and associated with innate immune responses; the IFN-α gene showed the highest up-regulation. Among the DETs uniquely expressed at 7 dpi., most of their host-viral interactions remain uninvestigated in fish, but in mammals, their functions have been related to cancer, inflammation and tissue damage.

In the KO analysis, functional annotation rendered 340 DETs with fold changes of $\geq$2 or $\leq$0.5 found at both 3 and 7 dpi.. Of these, 58 pathways had DETs expressed at both 3 and 7 dpi. with different expression levels, and the most enriched in genes were those related to amino acid metabolism, inflammation, cellular and immunological processes. Otherwise, 32 DETS had pathways with different expression levels at 3 and 7 dpi., most up-regulated and related to metabolism. Also 170 pathways had genes uniquely expressed at 3 dpi. and 30 pathways had genes uniquely expressed at 7 dpi.. The KOs with the highest fold changes were expressed at 3 dpi. and were related to host defense and the induction of different antiviral stages.

Our results indicated that at 3 and 7 dpi., many immune-related genes were up-regulated. At 3 dpi., most of the expressed genes were related to innate immunity, such as several lLs and IFN-α; at 7 dpi., the most representative DETs were involved in tissue damage, inflammation, intracellular membrane trafficking and altered Na^+/Ca^+ exchange. Gene clusters of two types can be identified: one including genes with temporal expression patterns differentiating the immune responses of asymptomatic from symptomatic fish, and the other with clusters representing molecular signatures of unique and contrasting temporal dynamics. And finally, we have shown the involvement of some immune pathways during IHNV infection, such as TLR, JAK-STAT and apoptosis. However, to deeply understand the factors affecting the expression of these gene clusters requires further work.

V. ACKNOWLEDGMENTS

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Discusión.

La finalidad de los estudios biológicos consiste en descifrar y entender la complejidad de un sistema vivo a cualquier escala. Sin embargo, es necesario realizar estudios parciales partiendo de grupos para reducir la cantidad de información y facilitar su análisis. En el caso de esta tesis doctoral nos hemos centrado en estudios de vacunación en el modelo trucha arco iris, orientados hacia la expresión génica ante los estímulos de vacunas DNA orales e infecciones virales. Además hemos determinado la protección proporcionada por estas vacunas DNA en infecciones clínicas y subclínicas y las posibilidades de optimización en cuanto a administración. Para la primera consideración los principales factores a tener en cuenta son genes, RNAm, algunas proteínas y en menor medida, algunas funciones celulares. Para la segunda, la disminución de la mortalidad y de la aparición de portadores de virus así como la evaluación del alimento como vehículo de vacunación oral.

Para el desarrollo de esta tesis doctoral, en el Laboratorio de Virus de Peces del CIB se utilizó como modelo de experimentación *in-vivo* la trucha arco iris (*Oncorhynchus mykiss*), un teleósteo salmoniforme, para caracterizar la eficiencia de dos vacunas de DNA frente a los virus de IPNV e IHNV, que se administraron por vía oral. Además de los experimentos clásicos de mortalidad acumulativa y determinación de los porcentajes de supervivencia relativa (RPS) de cada una de las vacunas, se estudiaron las respuestas transcriptómicas generadas frente a los procesos tanto de vacunación como infección en varios órganos y tiempos post-estimulación. Para esto, los genes expresados se analizaron mediante la determinación de la intensidad de su expresión en el caso de los microarrays o la abundancia de las secuencias en el caso de la técnica de RNA-seq. En este último caso, tras la obtención de los genes diferencialmente expresados, se adscribieron a las categorías funcionales específicas mediante análisis ontológico “GO” o su localización en rutas metabólicas o pathways, incidiendo especialmente en la respuesta inmune del hospedador ante estos estímulos.

La vacuna DNA frente al IPNV administrada por vía oral fue desarrollada y caracterizada en el Laboratorio de Virus de Peces del CIB previamente a esta tesis doctoral, por lo cual ya contaba con trabajos que demostraban que la construcción pcDNA-VP2 era capaz de expresar la proteína VP2r del virus IPN en diferentes tejidos de los animales a los cuales se les había administrado el plásmido de expresión; y su capacidad para estimular la respuesta inmune innata, e inducir anticuerpos neutralizantes específicos frente al virus. Además de los ensayos de protección con la vacuna pcDNA-VP2 administrada por vía oral, los cuales dieron buenos resultados (RPS de 66 a 94 % dependiendo de edad de los peces e infectividad del virus) (de Las Heras et al. 2009; de las Heras et al. 2010). Por esta razón, el presente trabajo de Tesis Doctoral se origina a partir de estos conocimientos previos, dado que la tasa de protección alcanzada por la vacuna pcDNA-VP2 frente a la infección con IPNV permitía incidir en más aspectos sobre la inmunización proporcionada por la vacuna, explorar nuevos métodos de administración más adecuados para su uso en acuicultura, extender nuestros estudios a vacunas frente a otros virus; y teniendo en cuenta el escaso conocimiento de los efectos de las vacunas DNA orales en peces, además que en los últimos años hay un interés creciente por el estudio sobre funcionalidad y regulación del sistema inmunológico de peces teleósteos, que permitan la prevención de las patologías, la mejora de la sanidad en los cultivos y la disminución de pérdidas económicas; se plantea como primer paso a este estudio los siguientes pasos:

Objetivo 1: Caracterizar la expresión génica inducida en trucha arco iris por una vacuna DNA oral frente al virus de la necrosis pancreática infecciosa.

El objetivo se abordó con técnicas como microarrays y qPCR para el análisis transcriptómico de algunos genes de respuesta inmune. Los órganos inicialmente seleccionados fueron el riñón anterior (principal órgano hematopoyético y con relevancia inmunológica), y el ciego pilórico (órgano de contacto directo con la vacuna e implicado en inmunidad de mucosas). Estos estudios se llevaron a cabo a los 7 días post-inmunización mediante la técnica microarray de oligonucleótidos de trucha arco iris de 60-mer oligo 8x15K (ID032303) con 6442 genes (diseño de J.Coll).

Los resultados obtenidos con el microarray nos permitieron seleccionar varios genes inmunológicos estimulados por la vacuna, por lo cual el siguiente paso consistió en identificar la similitud o mimetización de la respuesta inmune generada ante la vacunación respecto a la infección viral. Para ello, se llevaron a cabo estudios comparativos de la cinética de expresión inducida por el virus IPNV y la vacuna en varios órganos de trucha arco iris. Los resultados obtenidos muestran una tendencia a la similitud en los perfiles de expresión génica. Los niveles de expresión génica fueron mayores en peces infectados que en vacunados, excepto para el caso del IFN-I que presentó mayores niveles de expresión en los peces vacunados, indicando que este gen puede ser clave en el efecto de protección que presenta la vacuna frente a este virus.

Debido a la vía de administración de esta vacuna (oral), el siguiente paso consistió en conocer la respuesta del tracto digestivo ante el estímulo de la vacuna, ya que este órgano presenta una actividad propia de inmunidad de mucosas que puede ser relevante para la captación, absorción y eficacia de la vacuna. El intestino fue dividido en cinco segmentos diferentes para determinar en todos ellos el tipo de estímulo y la rapidez con que se produce tanto por la vacuna como por la infección de IPNV. Se procedió también a la caracterización de quimioquinas inducidas y sus receptores, principales quimio-atrayentes de células del sistema inmunitario.

El tracto digestivo presenta un conjunto de órganos con una respuesta inmune bastante dinámica frente a distintos estímulos; en nuestro caso se observaron células y niveles de expresión génica de receptores moleculares propios de células B, siendo esta la primera descripción de estas células involucradas en la respuesta inmune tardía en el sistema digestivo y en grasa peritoneal a los 10 días post-vacunación.

En resumen, los pasos que seguimos para desarrollar este primer objetivo fueron:

Estudio de expresión génica en tejido renal y ciego pilórico de peces vacunados a los 7 días, mediante análisis de microarray de oligonucleótidos específico de trucha arco iris (Ballesteros, Rodriguez Saint-Jean, et al. 2012).

Estudio comparativo de patrones de expresión génica involucrados en la respuesta inmune temprana y tardía en truchas inmunizadas con pcDNA-VP2 y en truchas infectadas con IPNV, con el fin de identificar los genes responsables de la protección frente a la infección (Ballesteros, Rodríguez Saint-Jean, et al. 2012).

Expresión de quimioquinas y sus receptores en los diferentes segmentos de intestino de peces infectados con IPNV y en truchas vacunadas con pcDNA-VP2 a diferentes tiempos post infección y/o vacunación mediante qPCR e inmunohistología (Ballesteros, Rodríguez Saint-Jean, et al. 2013).

Determinación de la respuesta inmune tardía y receptores moleculares propios de linfocitos B en los diferentes segmentos del intestino de trucha vacunada oralmente (Ballesteros, Castro, et al. 2013).

A continuación se discuten los resultados más significativos obtenidos.

1.1. Este trabajo es la primera descripción de respuesta sistémica y de mucosa inducida por una vacuna DNA, pcDNA-VP2 específica para IPN y administrada por vía oral.

El estudio se realizó en dos órganos diferentes, riñón anterior y ciego pilórico a los 7 días post vacunación; se eligieron por ser el riñón anterior uno de los principales tejidos involucrados en la respuesta inmune de la trucha y el ciego pilórico, un órgano de contacto directo con esta vacuna oral y con la capacidad de generar una respuesta inmune propia de mucosas. Por otra parte, el tiempo de muestreo fue seleccionado debido a que en trabajos previos se había comprobado que a los 7 días post-vacunación es cuando se observaban los mayores niveles de expresión del transcrito VP2 de la vacuna, en el riñón anterior (de las Heras et al. 2010); otros investigadores también habían obtenido similares resultados al inyectar la vacuna por vía intramuscular (Purcell et al. 2006).

Los resultados demostraron que en ambos órganos se incrementa el número de genes regulados. Sin embargo, los patrones de expresión fueron claramente diferentes. Es en el riñón donde se obtuvo una respuesta transcripcional más intensa, 68.8% de los genes estaban estimulados comparando con 7,8% en el ciego pilórico, posiblemente debido a que algunas señales moleculares han sido estimuladas desde el ciego pilórico hacia el riñón anterior vía sanguínea, induciendo parte de esta respuesta; otra hipótesis puede ser que el ciego pilórico a los 7 días ya no es un órgano con una respuesta activa, debido a que las células involucradas en la respuesta inmune en el ciego pilórico podrían haber emigrado a los órganos internos, causando una disminución del nivel de expresión de la mayoría de las transcripciones en este órgano, si bien todavía no existen evidencias que demuestren fehacientemente alguno de estos dos casos hipotéticos.

Los genes estimulados o sobre-expresados en el riñón anterior, corresponden principalmente a genes inducibles por IFN relacionados con Mx, tales como Mx1, Mx2 y Mx3. Sin embargo en ciego pilórico sólo el gen Mx 3 esta estimulado. Estos resultados se corresponden con los descritos en vacunas DNA administradas por vía intramuscular, donde las tres isoformas de Mx están estimuladas en el riñón anterior y únicamente el gen de Mx3 aumenta en el sitio de inyección (músculo) (Tafalla et al. 2007). Estos resultados descritos en riñón sugieren que la expresión de éstas tres isoformas del gen Mx podrían ser necesarias para lograr efectos antivirales (Caipang, Hirono, and Aoki 2009; Lorenzen et al. 2009).

Es importante resaltar que en riñón y ciego pilórico de peces vacunados se encontraron transcritos estimulados que sugieren un papel relacionado con células dendríticas presentadoras de antígeno. Entre esos transcritos cabe destacar los genes CD11 y CD209 marcadores de células dendríticas, además del TLR5m el cual se localiza en la membrana de estas células; el gen Vig1, es inducido por el virus VHS en células dendríticas además de la IL12 en respuesta a patógenos intracelulares. La IL12 tiene especial interés debido a que ha sido descrita como posible candidato a adyuvante de vacunas. El incremento en la expresión de citoquinas pro-inflamatorias como IL12, IL8, IL11 se observó en riñón, pero al mismo tiempo se inhibió la expresión de IL1β en ciego pilórico, lo que indica una supresión del proceso inflamatorio temprano en este órgano.

De especial interés fue la detección por RTqPCR del incremento en la expresión de IgM e IgT en el ciego pilórico comparado con el riñón anterior, ya que indica una potencial implicación de la inmunidad de mucosa inducida por la vacuna.

De todos estos resultados se deduce que la protección observada a los 30 días post-vacunación fue precedida por una compleja y específica respuesta inmune que se inició tanto de forma general como localmente en órganos.

De los genes evaluados por hibridación a microarrays se seleccionaron algunos que presentaban niveles de expresión de 4 veces más que el control sin vacunar y que diferían significativamente de éste ($p<0,05$) con objeto de confirmar los resultados por RT-qPCR. Se seleccionaron 15 genes y se concluyó que con ambos métodos se obtienen niveles de expresión similares.

1.2. Vistas las diferencias a nivel transcripcional encontradas en los dos órganos evaluados en los resultados del microarray, parecía interesante conocer mejor la respuesta inmune inducida tras la vacunación y establecer comparaciones con las respuestas en truchas infectadas con IPNV. Con el fin de conocer si existe un paralelismo en la respuesta desencadenada por la vacuna y el virus, se plantearon series de experimentos en diferentes órganos de truchas vacunadas con (pcDNA-VP2) y muestreadas a diferentes tiempos, utilizando para la evaluación de la respuesta los perfiles de expresión de los 15 genes previamente seleccionados. Y se comparó con la respuesta transcripcional en truchas infectadas y procesadas a los mismos tiempos.

Los resultados obtenidos sugieren que los mecanismos por los que la vacuna induce protección frente al IPNV son similares a los mecanismos de defensa que se producen en el hospedador tras la infección con IPNV. En vacunas DNA orales la distribución del transgen (en nuestro caso el de la proteína VP2 del virus) en los diferentes órganos ha sido aún muy poco estudiada, ya que esta vía de administración en peces apenas se ha abordado. En el caso de vacunas de DNA por vía intramuscular en salmones se ha observado que el plásmido es capaz de detectarse en varios órganos, evitando la degradación en el sitio de administración y distribuyéndose a través del flujo sanguíneo (Tonheim, Dalmo, et al. 2008). Por otra parte, el riñón anterior puede contribuir a la conservación del plásmido, debido a que actúa como un órgano con células que favorecen la transfección y producción del inserto del plásmido, además de aislarlo del torrente sanguíneo evitando su degradación con lisozimas (Garver et al., 2005).

Según los valores relativos de expresión del gen de la VP2 en diferentes tejidos (riñón anterior, bazo, intestino posterior, ciego pilórico, branquias (timo) de alevines de trucha vacunadas con pcDNA-VP2 o infectadas con IPNV, concluimos que, a los 7 días post-vacunación o post infección, se detecta expresión de VP2 en todos los órganos estudiados en peces vacunados e infectados. En los órganos de peces infectados la expresión de VP2 fue entre 10 y 100 veces mayor que en los peces vacunados, como era esperable en una infección de IPNV. En los peces infectados los mayores niveles se detectaron en riñón e intestino, y en el grupo de los peces vacunados los niveles de expresión variaron menos (entre 2 y 7.7 valores medios relativos) en los diferentes órganos. Estos resultados confirmaron que las micropartículas utilizadas fueron adecuadas para proteger al plásmido vacunal de la degradación por el bajo pH y por las proteasas existentes en el intestino, permitiendo la entrada y distribución del plásmido a los otros tejidos (Joosten 1997). Puesto que la expresión de VP2, (que codifica la proteína inmunogénica del virus) en truchas infectadas es mucho más alta que en las vacunadas, sería lógico que la respuesta inmune en estas truchas también fuera mayor. Se trataba aquí de comprobar si los perfiles transcriptionales inducidos por la vacuna, administrada por esta vía, podían ser comparables con los del virus. Ello proporcionaría datos interesantes a la hora de juzgar la potencialidad de la vacuna y su posible optimización.

Uno de los resultados más significativos fue el inesperado alto nivel de expresión de muchos transcritos en el timo/branquia tras la vacunación. Una posible explicación es porque podría ser uno de los primeros tejidos en entrar en contacto con la vacuna, incluso cuando aún no está completamente liberada del encapsulamiento en alginato pero suficiente para que haya una respuesta más rápida. Sin embargo, la expresión de VP2 es menor que en otros tejidos, como por ejemplo el intestino. Otra explicación alternativa a estos altos niveles de transcritos es que puede deberse a una respuesta secundaria debida a señales emitidas por otros tejidos internos, como el riñón y ciego pilórico, en los que VP2 se está expresando más. Por otra parte se observó que, aun en menores niveles que los inducidos por el virus, la vacuna indujo expresión diferencial de IgM y de IgT en intestino y en branquias a los 7 días post-vacunación. Es interesante resaltar que las branquias pueden considerarse como un órgano de mucosa y que IgT es considerada como la inmunoglobulina especializada en respuesta inmune de mucosa. El descubrimiento de esta Ig, relativamente reciente, cambió el paradigma de que IgM es la única inmunoglobulina en peces (Zhang et al. 2010).

La expresión diferencial de los genes en riñón anterior en truchas vacunadas y en truchas infectadas con IPNV, evidencia una mayor expresión de todos los genes en los peces infectados respecto a los peces vacunados pero con perfiles de expresión similares. Como era esperable (Samuel 1991) son destacables los IFNs, las ILs inducidas, los genes de la ruta JAK-STAT y MHC, entre otros. Puesto que esta vacuna ha sido ampliamente probada y presenta una protección de alrededor del 80% (de las Heras et al. 2010) es deducible que los niveles de expresión observados son suficientes para otorgar protección adecuada frente a este virus. La vacuna imita al virus y logra con éxito suscitar una reacción de defensa en el hospedador. Es destacable que el IFN-I fue el gen con una expresión similar entre los peces infectados y vacunados, lo cual podría ser importante para inducir y/o generar la protección frente a la enfermedad; esto puede ser debido a que la vacuna de DNA únicamente contiene el gen VP2, mientras que el virus presenta dos genes que codifican dos proteínas (VP4 y VP5) que son capaces de evadir el sistema inmunológico de la trucha, reduciendo los niveles de IFN para favorecer la replicación viral (Skjesol et al. 2009).

En general, todos los niveles de expresión de los genes relacionados con el IFN (STAT1, IFN-I, IFN-γ, Mx1 y Mx3) son mimetizados en las truchas inmunizadas con pcDNA-VP2 respecto a las truchas infectadas; sugiriendo que la eficiencia de la vacuna pcDNA-VP2 por vía oral puede ser gracias a su capacidad para inducir esta respuesta

Los resultados obtenidos nos permiten una visión general de la respuesta inmune temprana de la trucha ante dos estímulos (vacuna y virus), por lo que estos datos pueden ser útiles para optimizar la vacuna y potenciar la respuesta de la trucha a este tipo de vacunación y vía de administración. Y dentro de estos resultados los observados en intestino planteaban interesantes hipótesis sobre posible modulación diferencial de genes en distintos segmentos del tracto digestivo. Para su estudio se diseñó el siguiente trabajo:

1.3. La mayoría de los trabajos que contemplan respuestas inmunes en intestino de teleósteos se han centrado hasta ahora en los segmento posteriores y apenas se han descrito en las otras zonas. En este trabajo nos propusimos comparar el efecto que produce la vacuna pcDNA-VP2 por vía oral y la infección con virus IPN en la regulación de varias quimioquinas y de receptores de quimioquinas que se relacionan con la respuesta inmune de mucosas, en diferentes segmentos del intestino de trucha arco iris.

Las quimioquinas constituyen uno de los primeros factores secretados por el sistema inmune tras el encuentro con un patógeno, el estudio de estas moléculas de señalización en las primeras líneas de defensa resulta interesante en la patogénesis viral de peces (Kerry J Laing and Secombes 2004).

Los niveles de transcripción de los genes que codifican para receptores de quimioquinas como CCR7, CCR9 y CCR9B, fueron evaluados debido a que están implicados en el reclutamiento de células inmunes en el intestino de los mamíferos (Jang et al. 2006; Richmond 2008) y que en teleósteos aún están poco estudiados. Las quimioquinas seleccionadas, CK9, CK10, CK11, CK12, CCR7 y CCR98, pertenecen al grupo de las quimioquinas CCL27/28 y CCL19/21/25, que tienen una homología filogenética y funcional similar entre mamíferos y salmónidos; su regulación como respuesta a otro rhabdovirus (VHSV) se ha visto también en tejidos de mucosa como piel y branquias (Montero et al. 2011). También incluimos en este estudio el complejo principal de histocompatibilidad (MHC-II) y el factor de necrosis tumoral α (TNF2α) como marcadores de la presentación antigénica e inflamación, respectivamente.

Cuando comparamos la respuesta inmune del intestino (5 segmentos) de los peces infectados con IPNV con la respuesta a la vacunación oral con pcDNA-VP2 observamos que la transcripción de las quimioquinas, receptores de quimioquinas, MHC y TNF se detectaba en todos los segmentos del intestino en ambos grupos, aunque con importantes diferencias y niveles de expresión. Las quimioquinas CK9, CK10, CK11 y CK12 estaban sobre-expresadas en la mayoría de los segmentos. En los peces vacunados con pcDNA-VP2 oral la expresión de los genes CK9, CK10 y CK12 estaba inhibida en el ciego pilórico de los peces vacunados. Todo ello puede indicar por una parte que los diferentes segmentos del intestino tienen distintas capacidades de reclutar leucocitos o también que el virus y la vacuna utilizan diferentes mecanismos inmunológicos. E incluso que virus y vacuna se expresen de forma diferente en los distintos segmentos; de hecho nuestros resultados indican que, tras la vacunación, la transcripcón de VP2 se detectó en todos los segmentos pero fue significativamente mayor en los tres primeros.

Estudios anteriores sugieren que las quimioquinas CK9, CK10, CK11 y CK12 regulan a nivel de mucosas la respuesta frente a varias infecciones virales en las branquias y piel de las truchas (Montero et al. 2011), presentando un perfil transcripcional similar a las quimioquinas asociadas a la respuesta de tejidos linfoides periféricos (Martinez-Alonso et al. 2012; Montero et al. 2009). También se ha postulado que la quimioquina CK9, puede estar relacionada con el aumento de la capacidad quimiotáctica en las células epidérmicas y/o endoteliales en repuesta a una infección (Montero et al. 2009).

En este trabajo se encontró un aumento en la regulación de los diferentes genes (quimioquinas, receptores de quimioquinas, MHC-II o TNF-α) en los cinco segmentos, sugiriendo que otras células además de las células B están jugando un papel importante en la inmunidad de la mucosa en estos segmentos (Trebble et al. 2003; Vestrheim, Lundin, and Syed 2007).

Entre los hallazgos más destacables de este estudio está la identificación, por primera vez, de linfocitos T $CD3^{+}$ en el intestino anterior, ciego pilórico, intestino medio y posterior de los peces infectados con IPNV. Nuestros resultados con virus estarían en la línea de un trabajo realizado también en trucha, con un parásito intestinal (*Ceratomyxa)* (Bernard et al. 2006) donde se describieron células T $CD3^{+}$ en leucocitos intra epiteliales (IELs) del intestino posterior. Algunos aspectos de este trabajo de quimioquinas y sus receptores en los diferentes segmentos del intestino tienen especial interés debido a la falta de marcadores específicos celulares en trucha. Nuestros resultados proporcionan datos para la mejor comprensión del sistema inmune en peces, ya que pueden indicar de forma indirecta la participación y reclutamiento de células como NK o dendríticas; estas quimioquinas como por ejemplo la CCR7, se expresan en células dendríticas maduras del tracto digestivo de mamíferos (Jang et al. 2006; Sallusto et al. 1998).

En resumen, en trucha arco iris la infección con virus IPN por inmersión modula la transcripción de varias quimioquinas como CK9, CK10, CK11, CK12, CCR7, CCR9, CCR9B, MHC-II y TNF-γ, al igual que moviliza células IgM^+, IgT^+ y linfocitos T $CD3^+$ en diferentes segmentos del tracto intestinal. Además, las células epiteliales ubicadas en los diferentes segmentos del intestino, responsables de la producción de quimioquinas son estimuladas de diferentes maneras a lo largo del tracto intestinal, ya sea por el virus IPN como por la vacuna pcDNA-VP2.

Otros autores han demostrado que la inmunoglobulina IgT no es exclusiva de mucosas (Castro et al. 2011); en nuestro trabajo en el tracto digestivo también se observa IgM en una cantidad significativa; por otro parte, la IgT también ha sido encontrada en el bazo y plasma de peces infectados con virus (Martinez-Alonso et al. 2012).

Tanto la vacuna de DNA “pcDNA-VP2” por vía oral como el virus IPNV tienen la capacidad de estimular una respuesta inmune completa en los diferentes segmentos del tracto intestinal; en el caso de la estimulación con el virus IPN se observó la secreción de IgM^+ e IgT^+ (propia de inmunidad de mucosas) a lo largo del intestino. Sin embargo, la replicación del virus IPN corresponde a un evento más complejo respecto a la vacuna de DNA frente a la respuesta generada en el tracto digestivo, ya que el virus además de provocar una respuesta inmunológica rápida también genera lesiones o daños a los tejidos. Además, el virus presenta otras proteínas estructurales diferentes a la proteína VP2, que son importantes en la patogénesis viral (Pedersen et al. 2007).

Tras detectar y describir la respuesta inmunológica observada en los diferentes segmentos del intestino de truchas vacunadas, parecía interesante profundizar en su respuesta inmunológica celular algo más tardía, a los 10 días post tratamiento. El objetivo era observar la distribución en todo el tracto intestinal de posibles células B mediante inmunohistoquímica y determinar la transcripción de genes codificantes de inmunoglobulinas y factores de transcripción como Blimp1 y Pax5 implicados en la maduración de las células B, mediante RT-qPCR.

Los resultados obtenidos se discuten a continuación y se plasmaron en el trabajo:

1.4. Las células o linfocitos B son leucocitos básicos para la inmunidad mediada por anticuerpos con activación específica de fijación de antígeno. Estas células en mamíferos presentan varias etapas de maduración, en las cuales se han encontrado varias inmunoglobulinas como IgM, IgT e IgD en su superficie o incluso en algunos estadios en su citoplasma (McHeyzer-Williams and McHeyzer-

Williams 2005). Los genes correspondientes a Blimp1 y Pax5 son útiles en el estudio de las células B a lo largo del tracto digestivo de la trucha, ya que Pax5 es un factor de transcripción expresado en las células B y es sub-expresado cuando se produce la maduración de las células B debido a la inducción del gen Blimp1, el cual es un represor transcripcional.

En nuestro trabajo, entre los primeros resultados, se observó que, respecto a respuestas de células B el ciego pilórico fue el segmento del intestino más activo inmunológicamente; esto podría estar relacionado con su alta capacidad de adsorción y la presencia de leucocitos intracelulares IgM^+ e IgT^+.

Los niveles de transcripción de los genes IgM, IgT e IgD a lo largo del tracto digestivo de truchas vacunadas con la vacuna DNA-VP2 encapsulada en alginatos y la transcripción de los factores de transcripción Blimp y Pax5 se determinaron en los distintos segmentos del tracto digestivo, asi como en truchas control. En estas últimas se identificaron células B IgM^+ e IgT^+ en todos los segmentos de intestino a excepción del estómago. A nivel de inmunohistoquímica, se observó que las células B IgM^+ se encontraban en la lámina propia y las células IgT^+ fueron localizadas en linfocitos intraepiteliales (IELs). En peces vacunados por vía oral, observamos un aumento en el número de IELs en el ciego pilórico; de hecho fue el único órgano con estimulación significativa. También se detectó regulación de genes de IgM, IgT, IgM de membrana y del factor de transcripción Blimp1; y sus niveles de expresión se correlacionaban con la transcripción del antígeno VP2 de la vacuna.

Este trabajo es el primer estudio donde se observan células B IgM^+ e IgT^+ en IELs, sugiriendo que las células B pueden encontrarse como IELs en otros tejidos de mucosas como se ha descrito que ocurre en IELs de las glándulas adenoides y amígdalas de mamíferos, tejidos ricos en células B (Boyaka et al. 2000).

De acuerdo con los resultados observados en los trabajos acerca de la IgT^+ en los diferentes segmentos intestinales, se podría sospechar que en etapas tempranas de estimulación, se observa una respuesta conjunta tanto de IgM^+ como de IgT^+ que podría incluso regularse de formas distintas según el tipo de inmunización, o alternativamente, que la inmunoglobulina IgT^+ plasmática actúe en una fase más temprana y luego sus niveles decrezcan, dejando casi en exclusividad a las células IgM^+ en las fases más tardías.

Además, con el fin de conocer los niveles basales propios del tejido de células B exclusivamente para estos genes, sin que existiese interferencia de la expresión producida por los leucocitos de sangre periférica (PBLs), se determinaron cuantitativamente los niveles de expresión de los factores de transcripción Blimp1 y Pax5 en peces perfundidos control. Los resultados indicaron que a expresión de los genes Pax5 y Blimp1 no se correlacionaban y que Pax5 se estimulaba 1000 veces más en PBLs que en cualquier segmento del intestino. De ello se deduce que la sobre-expresión detectada en el ciego pilórico en truchas vacunadas podría ser debida a un aumento de las células plasmáticas, y por ende, los altos niveles de expresión observados serían explicables por la expresión en estas células, fácilmente difundidas por la alta irrigación de este tejido. Por el contrario, el incremento en la expresión de

Blimp1 podría indicar la maduración local (en el propio tejido intestinal) de las células plasmáticas como respuesta a la vacunación.

Un hallazgo interesante en nuestros ensayos de inmunohistología, fue la detección de células B en el tejido adiposo que rodea el ciego pilórico, un hecho no descrito en teleósteos. Este tejido adiposo rodea el ciego pilórico y nuestro estudio es el primero que asigna un rol inmunológico al tejido adiposo visceral de peces. Este hecho se había descrito en tejido adiposo humano y de mamíferos (Kaminski and Randall 2010). El tejido adiposo en los mamíferos se separa generalmente en el tejido adiposo visceral y subcutáneo, siendo el tejido adiposo visceral el que es metabólicamente e inmunológicamente más activo (Yang et al. 2008). En los mamíferos, en el omento mayor se han descrito macrófagos y células B B (Van Vugt et al. 1996), al igual que células dendríticas (Bedford et al. 2006) y células NK (Lynch et al. 2009), sin embargo, apenas se conocen estudios que hayan demostrado una respuesta de las células inmunes asociadas de grasa tras la inmunización por vía oral (Rangel-Moreno et al. 2009). Por lo cual estos resultados apuntan a un papel importante de las estructuras linfoides asociadas a la grasa, tanto en la respuesta inmune innata como adaptativa en los teleósteos.

Con todos nuestros experimentos anteriores habíamos demostrado que la vacuna pcDNA-VP2 oral frente al IPNV modula series de genes que producen una compleja respuesta inmune, que esta respuesta sigue los perfiles de la que se produce en principio tras la infección con el virus, y que las respuestas a lo largo del intestino son diferenciales; además hemos comprobado el protagonismo de células B inmunidad ligada al tejido adiposo. Todo ello corroborando que la vacuna DNA puede seguir activa tras su paso por el digestivo, en una administración oral. Sin embargo, la administración oral en estos primeros ensayos se había realizado de forma individual, para garantizar que todas y cada una de las truchas recibía la dosis correcta. El siguiente paso lógico consistió en encontrar un método efectivo, fácil, que no requiera manipulación individual y que permitiese que la vacuna pueda suministrarse por vía oral de forma masiva Para lograrlo se abordó el objetivo 2º de esta tesis doctoral.

Objetivo 2: Optimizar métodos de administración de vacuna DNA oral

Este objetivo se encuentra desarrollado en el trabajo publicado en *Fish Shellfish Immunol.* 2014 (Ballesteros, Rodriguez Saint-Jean, and Perez-Prieto 2014).

Sabemos que la vacuna pcDNA-VP2 administrada por vía oral de forma manual e individualmente utilizando una punta de pipeta, es capaz de estimular al sistema inmune innato y adquirido de la trucha y lograr un RPS del 80% frente a la infección por IPNV. Quisimos determinar si era posible utilizarla en el alimento sin perder eficacia. Para administrarla con menor manipulación al pez y así disminuir su estrés, se incorporó esta vacuna directamente al pienso de los peces; para ello, la vacuna pcDNA-VP2 recubierta con alginato de sodio fue administrada mezclada con el pienso durante 3 días consecutivos y tras 15 y 30 días contados a partir del último día de consumo, los peces fueron infectados por inmersión con el virus IPN. Comprobamos que la vacuna (pcDNA-VP2) administrada con el alimento fue capaz de llegar hasta los órganos internos, encontrándose niveles similares de expresión del gen VP2 a los detectados al administrar la vacuna directamente en la boca del pez con una pipeta. Este resultado es novedoso ya que es el primero en demostrar que una vacuna DNA frente a IPNV puede administrarse incorporada al pienso sin perder efectividad.

Por otra parte, a los 30 días post-infección se comprobó que la vacuna administrada en el alimento fue capaz de proteger a los peces de la infección por IPNV; la mortalidad observada en el grupo control infectado fue alta mientras que durante ese periodo de tiempo, en los peces vacunados la mortalidad fue de solo del 14 %, con un RPS de 86%, En los controles realizados con la vacunación individual con pipeta, la protección fue algo menor (alrededor del 76%)

En comparación con los resultados obtenidos en estudios previos con esta vacuna administrada por vía oral con pipeta, se observó aquí un aumento en la expresión de genes inmunes de respuesta innata y una posible explicación sería la mayor dosis suministrada ya que los peces fueron alimentados con pienso “medicado” durante 3 días consecutivos, con un consumo a voluntad, mientras que con pipeta se les administró una única vez. Además de la alta estimulación de IFN-I y otros genes marcadores de inmunidad inespecífica, la expresión de otros que modulan la citoquinas pro-inflamatorias (como IL12 o IL8) también se incrementó significativamente en distintos tiempos; esta regulación era interesante porque estos genes se consideran reguladores de respuesta inmune, representando una conexión entre respuesta innata y adaptativa.

Además en este trabajo se incluyó también el estudio de los genes de diferenciación funcional de linfocitos (CD4 y CD8) y comprobamos que el propio inserto de la vacuna, el gen de la proteína VP2, es capaz de estimular y/o activar los linfocitos T tanto citotóxicos como helper, por lo cual esta respuesta puede ser necesaria para aumentar de forma significativa la transcripción de genes relacionados con inmunoglobulinas y así generar protección ante la enfermedad. Consecuentemente, se detectaron en los peces vacunados títulos medios y altos de anticuerpos neutralizantes a los 30 días pv, que aumentaron hasta casi duplicarse a los 60 días pv. Este resultado es relevante porque entre los escasos experimentos de vacunas DNA orales en peces, los abordados frente a otro virus, IHNV, no dieron resultados positivos (Adomako et al. 2012).

En las truchas supervivientes se determinó la carga viral utilizando como marcador la detección por PCR del gen VP4 del IPNV, para evaluar también así la actividad de la vacuna. En los peces vacunados con pcDNA-VP2 en pienso e infectados los niveles de expresión de este gen fueron muy bajos, significativamente distintos a los hallados en truchas supervivientes del grupo control virus. Aun así, esto no implica necesariamente la presencia de viriones infectivos, ya que se realizaron pruebas de aislamiento viral del riñón anterior de todos estos peces en monocapas celulares sensibles a la infección con IPNV, y no se detectaron efectos citopáticos (ECP) en las muestras del grupo de vacunados e infectados, mientras que en el grupo de infectados se observaron ECP en todos los individuos muestreados.

En resumen, tras la comprobación de que la administración en pienso de esta vacuna era viable, se observó no solo inmunomodulación de genes marcadores, sino también una alta y específica protección a los 30 días pv con valores similares a los obtenidos con la vacuna pcDNA-VP2 recubierta con alginato de sodio y administrada por vía oral con pipeta, a pesar de la posible variabilidad del consumo de alimento a lo largo de los 3 días de vacunación.

El planteamiento siguiente fue determinar si, pese a los buenos resultados obtenidos con la microencapsulación del plásmido en alginato, sería posible mejorar su eficiencia (RPS 86%), probando otro tipo de recubrimiento; para ello, seleccionamos el chitosan.

Como parte de la optimización de los métodos de administración de la vacuna planteados en el objetivo 2 se sustituyó el recubrimiento de únicamente alginato por el recubrimiento de chitosan-alginato debido a su mayor estabilidad en medios ácidos como puede ser el tracto digestivo de los peces. Para el recubrimiento del chitosan-alginato se utilizan dos métodos diferentes de preparación de las microesferas, que se han descrito anteriormente en la sección 2.

Según los resultados obtenidos, este recubrimiento de alginato-chitosan mantuvo los niveles de protección altos, aunque no superó los obtenidos con el recubrimiento de alginato, no se observaron diferencias estadísticamente significativas en cuanto a la estimulación de genes relacionados con interferones e interleuquinas. Una posibilidad poco explorada en vacunas de peces es el tamaño de las partículas administradas, pues se ha señalado que partículas pequeñas (<100 nm) son más efectivas en la internalización en la célula, además presenta una mayor tasa de transfección (Prabha et al. 2002). Los mecanismos implicados en la captación y presentación de antígenos todavía no se entienden completamente para la vacunación oral en peces.

En mamíferos se conoce la importancia del diámetro de las microesferas en la encapsulación de medicamentos, los cuales suelen ser más pequeños que 10 micras (Agnihotri, Mallikarjuna, and Aminabhavi 2004; Jenkins et al. 1994; Rinaudo 2006; Shalaby 1995). Las micropartículas (diámetros de hasta 50 micras) pueden ser empleadas en la vacunación de peces con éxito, sin embargo, parece mejorar la eficiencia al suministrar partículas más pequeñas (Desai et al. 1996; Harikrishnan et al. 2012; Richardson 1999; Sinha et al. 2004; Sriamornsak, Burton, and Kennedy 2006; Thanou et al. 2002). El tamaño de micras en las partículas hace que puedan interactuar con membranas biológicas de una forma completamente nueva, induciendo de este modo respuestas no reportadas en tamaños más grandes; por lo cual se requieren estudios para conocer los posibles efectos secundarios potenciales. En nuestro caso, las microesferas utilizadas de alginatos tenían un diámetro de 5 a 10 micras.

La continuación experimental de la encapsulación en alginato/chitosan podrá ser una interesante propuesta para futuros trabajos, en los cuales se podría modificar los tamaños de las encapsulaciones y determinar así su influencia en la optimización de la vacuna.

Según los resultados obtenidos y al no observar una mejora significativa en la eficiencia de la vacuna y contando además con la ventaja de la sencillez de preparación, se optó por mantener la encapsulación únicamente con alginato para los demás estudios llevados a cabo en esta tesis, como el estudio de persistencia vírica en los peces vacunados con pcDNA-VP2 por vía oral, incluido en el objetivo 3 de este trabajo.

Objetivo 3: Determinar la eficacia de la vacuna pcDNA-VP2 oral en la prevención de estados de persistencia del virus de la necrosis pancreática infecciosa.

El siguiente objetivo se desarrollo en el trabajo aceptado en *Veterinary Immunology and Immunopathology 2015.*

La vacuna ideal frente a IPNV debe proteger a los peces en etapas tempranas de su vida, ser fácil de administrar y prevenir la formación de portadores; por esta razón en el objetivo 3 nos propusimos averiguar si la vacuna pcDNA-VP2 por vía oral además de proteger en alto porcentaje a los peces vacunados frente a la infección con IPNV, era capaz de evitar que los animales supervivientes fueran portadores del virus.

Es bien conocido que supervivientes a la infección por IPNV pueden ser portadores del virus que a su vez pueden transmitirlo a otros peces de la instalación y a las siguiente generación (Santi et al. 2004; K Wolf 1988). En consecuencia, para controlar las enfermedades virales en piscifactorías es importante evitar la formación de portadores

En este trabajo se demostró que en las truchas que habían sido vacunadas e infectadas y que eran supervivientes asintomáticos a la infección con IPNV, la infectividad y la presencia del virus habían desaparecido a los 45 días post infección. No se aisló virus en cultivos celulares inoculados con homogeneizados de órganos (riñón, hígado, bazo, intestino y branquias) de los supervivientes vacunados e infectados pero si en los cultivos inoculados con homogeneizados de órganos de peces infectados control. También se detectó la proteína VP2 del virus por IFI cuantificada por citometría de flujo. La carga viral se determinó mediante la valoración por RTqPCR de los niveles de expresión del gen VP4 del IPNV en los diferentes órganos determinando que fue significativamente menor en peces vacunados e infectados que en los controles infectados sin vacunar.

De acuerdo con estos resultados podríamos concluir que la vacuna evita la formación de portadores. La inducción de diversos genes inmunomoduladores provoca una inhibición de la replicación viral, como viene avalado por los resultados de RTqPCR, en donde se observaron altos niveles de transcripción de IFN-I a los 45 dpi en los peces vacunados e infectados, en comparación con los peces únicamente infectados. Por el contrario los niveles de IFN-γ, considerado marcador de inmunidad adaptativa, fueron bajos. Esto se ha descrito también en salmones supervivientes a una infección por IPNV (Marjara et al. 2011).

En la infección viral, la respuesta inicial del sistema inmune es la expresión de IFN-I, que induce un estado antiviral en las células para impedir la propagación del virus (Robertsen et al. 2003); en el caso de nuestra vacuna, esta modulación puede ser fundamental para evitar el estado de portador.

Se evaluaron también los niveles de expresión de otras citoquinas y su correlación con la capacidad de los peces vacunados de establecer una respuesta inmune humoral y celular contra el virus, con objeto de conocer si los mecanismos de inmunoregulación pueden influir en el fenómeno de persistencia viral.

En los peces supervivientes a la infección con IPNV se examinó la expresión de IFN-I, IFN-γ, TNF-α, IL8, IL10, IL12, MHC-I, IgM e IgT durante una infección en estado agudo (7 días) y en estado persistente (45 días). Los peces vacunados supervivientes a la infección con IPNV presentaron niveles de expresión de las citoquinas pro-inflamatorias, IL8 e IL10, significativamente más bajos que los peces supervivientes no vacunados. Esta observación corrobora las descritas en otros estudios (Wilson et al. 2011) que, en infecciones con otros patógenos parásitos observaron que la respuesta inmunoreguladora de la citoquina IL10 puede impedir el desarrollo de resistencia o inmunidad que se crea después de la enfermedad natural o después de la vacunación. Además en el estado agudo de infección por IPNV, los vacunados y sin vacunar presentan un aumento en la expresión IL8 al igual que lo observado por (Reyes-Cerpa et al. 2012). Sin embargo, los niveles de expresión del gen IL10 en estos peces fueron significativamente más bajos. IL 10 es una potente citoquina anti-inflamatoria descrita en mamíferos e IL8 es una quimioquina que induce activación endotelia, para reclutar células inmunocompetentes. El incremento de la expresión de IL10 se acompaña de un fallo para inducir IL 8 e IL1b.

La estimulación o sobre expresión de esta citoquina (IL8) puede estar relacionada con la pérdida de actividad de las células T (Young et al. 2007) o con los efectos inmunosupresores de la infección por IPNV, y pueden contribuir a la infección persistente. Incluso, recientemente se propuso que la fuerte inducción de esta citoquina en el riñón anterior puede ser utilizada como marcador de peces portadores (Reyes-Cerpa et al. 2012). Por lo cual, se puede especular que la actividad de la vacuna VP2 contribuye a la disminución en la carga viral de las células renales y, por tanto, a la baja regulación de la respuesta anti-inflamatoria como se puede observar en la inhibición de la citoquina IL10. De esta forma, la expresión de citoquinas anti-inflamatorias producidas en respuesta al virus IPN podría estar asociada al desarrollo de una débil respuesta inmune celular y humoral que podría explicar la supervivencia del virus a bajos niveles que permitan el establecimiento del estado portador asintomático o infección persistente. La inducción de este perfil de citoquinas podría ser parte de un posible mecanismo de evasión inmune.

Respecto a las inmunoglobulinas IgM e IgT, la vacunación no estimula la expresión a mayores niveles de los que induce el virus en el grupo control no vacunado (portadores también), sin embargo la vacuna imita la actividad que el virus induce y contribuye al mantenimiento de estas inmunoglobulinas a niveles apreciables en los supervivientes asintomáticos. En otros casos, de infecciones de virus en salmónidos como el virus de la septicemia hemorrágica (un rabdovirus), se ha descrito que tanto la IgM como IgT se encuentran regulados en las fases iniciales de la infección, ya que las células IgM^{+} e IgT^{+} participan en la respuesta antiviral (Aquilino et al. 2014). Sin embargo, es difícil determinar que parte de la actividad inmunológica está siendo inducida por el escaso virus que pudiera persistir en los peces vacunados supervivientes o por el gen VP2 propio de la vacuna administrada, debido a que el gen VP2 sigue expresándose en varios órganos como ha sido demostrado por (Rodriguez Saint-Jean, de las Heras, and Perez Prieto 2010). En las infecciones persistentes, los genes involucrados en la degradación de las proteínas virales se encuentran activados. Aunque se demostró la inhibición en la traducción, sugiriendo una participación en el control de la replicación del virus IPN y por ende, un papel en el mantenimiento de un número bajo de partículas virales en las células persistentemente infectadas (Marjara et al. 2011).

La vacuna pcDNA-VP2 disminuyó la expresión del gen no estructural VP4 del virus IPN, que sirve como un marcador de la infección viral. Por lo tanto, puede ser que a medida que la carga viral disminuye en el huésped, la actividad de genes de respuesta inmune inducidos por la vacuna puede contribuir de forma optimizada en la eliminación de los viriones restantes, eliminando el estado de portador.

En resumen, los experimentos aquí descritos acerca de la vacuna de (pcDNA-VP2) frente al virus de IPN ofrecieron resultados bastantes interesantes y novedosos, pues se ha contribuido al estudio del mecanismo de funcionamiento de una vacuna de DNA por vía oral y al estudio de la respuesta inmune en trucha arco iris ante un estímulo (vacuna y/o virus) en varios órganos en los que la respuesta era aún poco conocida cabe también destacar especialmente el éxito en la protección ante la enfermedad de una vacuna suministrada en conjunto con el pienso, que además es capaz de disminuir la carga viral de los peces infectados, lo que puede contribuir a impedir la persistencia vírica y la aparición de peces portadores en una instalación. En las piscifactorías que cierran el ciclo biológico de la trucha y mantienen sus propios reproductores, es relevante cualquier práctica que ayude en la prevención de la transmisión horizontal y vertical de esta enfermedad.

Objetivo 4: Evaluar la respuesta inmune y protección inducida por una vacuna DNA oral frente al virus de la necrosis hematopoyética infecciosa.

Este objetivo se desarrollo dentro del trabajo próximo a enviar a *Fish and Shellfish Immunology* 2015.

La alta eficiencia y protección de la vacuna frente al virus IPN al administrarla por vía oral ha sido la base para probar por esta misma vía la vacuna frente al virus IHNV (pIRF-G). Como mencionamos anteriormente se ha utilizado el plásmido que codifica el gen de la proteína de superficie G del IHNV en el que se reemplazó el promotor del CMV por el IRF1A específico de salmónidos. En el trabajo de tesis, se probó comparativamente por primera vez la eficacia de la vacuna pIRF1A-G administrada por vía oral e intramuscular frente al IHNV; además, con el fin de evaluar la respuesta inmune y protección inducida por la vacuna, se analizaron los mecanismos inmunológicos no específicos y específicos relacionados con la actividad antiviral. Tras la vacunación oral con la dosis menor (10 µg) se detectó expresión del transgen G en todos los órganos examinados. Esto indica no solo que las micropartículas de alginato protegen eficazmente la vacuna, evitando, al menos en parte, su degradación en el tracto digestivo, sino que traspasa el epitelio intestinal y es transportada por la sangre, alcanzando diversos órganos internos y externos del pez.

Posteriormente se determinó la producción de anticuerpos neutralizantes frente al virus a los 30 y 45 días post inmunización y se evaluó la protección que proporcionó la vacuna. Todos estos ensayos fueron realizados administrando diferentes dosis de vacuna (pIRF1A-G) por vía oral y además se compararon los resultados con los obtenidos al suministrarla por vía im. a una dosis única de 5 µg.

Los niveles de expresión del gen G fueron detectados en todos los órganos de los peces vacunados por vía oral, incluso en la menor dosis. Sin embargo era esperable que el incremento de la concentración de pIRF1A-G aumentase también la expresión del transcrito G del virus. El recubrimiento de alginato proporciona alta resistencia al pH ácido del estómago y favorece su liberación en el intestino medio y posterior (a pH 7 y 8.3, respectivamente). Pese a ello, es factible suponer algún deterioro a lo largo del tránsito por el digestivo, y nuestros resultados indican que la administración por vía oral necesita concentraciones de vacuna 20 veces mayor que la utilizada por vía im. Los órganos con los niveles más altos de expresión de este gen fueron el riñón anterior y el bazo y en ellos se evaluó la expresión de genes inmunológicos. Los resultados mostraron claramente un efecto dosis dependiente tanto en la estimulación de genes de la respuesta inmune como en protección frente al virus. La dosis más baja (10 µg) por vía oral fue capaz de estimular la expresión de los genes IFN-I, TLR-7 e IgM en el riñón, además de la inducción significativa de los genes Mx1, Vig1, Vig2 y TL-7 a los 3 días pv. en el bazo. Por el contrario, la expresión de los genes Mx1, Vig1, Vig2, TLR-3 y TLR-8 sólo fue detectada en los peces vacunados con dosis altas (50 y 100 µg). Por otra parte, estas dosis vacunales de 50 y 100 µg indujeron bajos niveles de anticuerpos anti-IHNV, con un RPS de 45 y 56% respectivamente.

Nuestros resultados indican que las expresiones de TLR-3, TLR-7 y TLR-8 fueron moduladas por la vacuna administrada tanto por vía oral como intramuscular. Los TLRs son proteínas de transmembrana que reconocen estructuras conservadas de patógenos, y en peces se ha descrito su regulación en infecciones virales (Palti 2011; Robertsen 2006). Por tanto implicados en la infección por IHNV. Curiosamente la administración de la vacuna oral a la dosis mayor (100 µg) indujo niveles de expresión de estos TLRs mas altos que los inducidos por la vacuna im., sugiriendo que esta vacuna mimetiza la respuesta transcripcional inducida por el virus. En nuestro trabajo con la vacuna oral para IPNV también se observaba este tipo de tendencia en la imitación de la respuesta inmunitaria generada por la vacuna en comparación con el virus (Ver trabajo (Ballesteros, Rodríguez Saint-Jean, et al. 2012) presentado en el objetivo 1).

No obstante la expresión de IFN-I y Vig2 detectada a los 3, 7 y 15 días pv en riñón y bazo de truchas vacunadas, fue similar, independientemente de la ruta de administración utilizada. Sin embargo otros trabajos han señalado que la administración oral de una vacuna estimula respuestas inmunes diferentes a las inducidas por antígenos administrados oralmente (Huttenhuis et al. 2006; Rombout et al. 2011). En nuestro caso la vacunación oral con 100 µg indujo en riñón niveles de expresión de CD4 (marcador de células T “helper”) y CD8 (marcador de linfocitos citotóxicos) mayores que la vacuna im. en riñón, lo que apoyaría la idea de que rutas diferentes pueden estimular respuestas inmunes celulares diferentes. Además observamos también modulación diferencial de IgM e IgT tras 15 días de la vacunación intramuscular u oral con 100 µg en riñón y bazo.

Según nuestros resultados, la dosis por vía oral a 100 µg y la vacuna por vía im. fueron las únicas inmunizaciones que demostraron regular la expresión de genes involucrados en la respuesta inmune adquirida como el gen CD4 tanto en el riñón anterior como en el bazo. Respecto a la cinética de la expresión génica de las células T tanto $CD4^+$ como $CD8^+$ se han demostrado una correlación con la protección en los salmónidos, indicando que otras partes de la respuesta inmune adaptativa como la acción de las células T citotóxicas Th1 pueden contribuir a la eficiencia de la inmunización (Munangandu et al. 2012). La expresión del gen IgM en ambos órganos fue correlacionada con la producción de anticuerpos, incluso a la dosis más baja de inmunización por vía oral; sin embargo, el nivel parece ser insuficiente para alcanzar altas tasas de protección cuando la dosis de la vacuna es baja por vía oral.

En cuanto a la protección frente a la infección otorgada por las diferentes dosis vacunales en la administración oral, los resultados mostraron también dosis dependencia, pues a medida que se incrementaba la concentración del plásmido se observaron mayores RPS. Y las diferencias entre las mortalidades en truchas vacunadas oralmente con 10, 20, 25 y 50 µg y las mortalidades en truchas no vacunadas o vacunadas con el plásmido vacío, fueron estadísticamente significativas. A la dosis más alta aquí estudiada (100 µg) el RPS fue únicamente un 10% más bajo que el observado tras la administración por vía im. Estos resultados son alentadores ya que proveen información acerca de dos estrategias diferentes de administración de vacunas DNA y sus diferencias en la estimulación de genes inmunológicos relacionados con la protección. Hay que considerar también que en nuestros experimentos el virus utilizado fue muy virulento, con mortalidades del 100%, comparando con otros descritos en la literatura (entre 60 y 80% de mortalidad), por lo que nuestros índices de supervivencia relativa son menores de lo que cabría esperar. Las diferencias en la protección inducida por la vacuna oral o im. podrían deberse también a un problema relacionado con la cantidad de antígeno inicial y la persistencia a través del tiempo para mantener la estimulación inmune suficiente.

En otros trabajos sobre las vías de administración de las vacunas DNA frente al IHNV con el inserto del gen G, la vacuna fue recubierta con PLGA (ácido D, L-láctico coglicólico) y administrada a través del pienso en los peces. La protección lograda fue baja, un RPS=22% utilizando una dosis de 43 µg por trucha arco iris de 5 g de peso; sin embargo, cuando esta misma vacuna fue administrada por vía im., el incremento en el RPS fue significativo (RPS=83%) (Adomako et al. 2012). Estos resultados indican que los recubrimientos tanto de alginato como de PLGA parecen ser adecuados en el recubrimiento de vacunas DNA para la administración por vía oral.

Nuestros resultados fueron mejores que estos, seguramente por el tipo de recubrimiento, pero aún se requieren más ensayos de optimización. Una aproximación podría ser incrementando la inmunogenicidad de la vacuna mediante su administración con adyuvantes para mucosa, tales como glucanos, o sales de aluminio; otra posibilidad sería incorporar plásmidos que codifiquen citoquinas coadyuvantes. A demás, si esta vacuna pudiese incorporarse al pienso y mantener su actividad, tal como sucede con la vacuna DNA oral para IPNV (analizada en el objetivo 1, (Ballesteros et al. 2014)) los peces podría vacunarse en días sucesivos, mejorando la dosis administrada y por ende la protección.

Objetivo 5: Determinar masivamente los perfiles de transcripción de trucha arco iris en respuesta a la infección con el virus de la necrosis hematopoyética infecciosa, o a una vacuna DNA.

Este objetivo se desarrollo dentro del trabajo próximo a enviar a *Veterinary Research* 2015.

Debido a que la vacuna DNA frente a IHNV por vía oral no presenta una capacidad similar de protección a la observada con la vacuna DNA frente a IPNV también por vía oral, quisimos conocer y comprender la respuesta inmune llevada a cabo por el virus IHNV en la trucha arco iris, con el fin de facilitar nuevos enfoques y medidas para el diseño de vacunas preventivas. Para ello se identificaron genes cuya expresión es alterada debido a la infección con IHNV, y por lo cual podrían estar involucrados en vías bioquímicas o pathways esenciales para la prevención de la enfermedad.

El presente estudio tiene como objetivo proporcionar un acercamiento a los eventos transcripcionales inducidos en la trucha después de la infección con IHNV para establecer una visión global de los cambios patológicos en dos etapas diferentes de la infección, en un periodo inicial de la infección cuando todavía no se detectan síntomas clínicos (3dpi.) y finalmente, cuando la enfermedad es evidente al observar signos clínicos y comienzo de la mortalidad (7dpi.)

El órgano seleccionado para este estudio fue el riñón anterior, debido a que es el principal órgano hematopoyético en trucha y, además, es uno de los órganos diana en la replicación de este virus.

Una vez se obtienen los resultados de los transcritos o genes implicados en alguna fase de la infección, el siguiente paso consiste en la descripción de las redes reguladoras y la coexpresión de grupos de genes implicados en rutas génicas o pathways implicados en los procesos fisiológicos, en nuestro caso, queremos enfatizar en la respuesta inmunitaria frente a los diferentes estadios de la infección. Lamentablemente, debido a los altos costes metodológicos de la secuenciación transcriptómica, su aplicación es restringida a un selecto número de individuos, tejido y tiempos (cinética de la infección), por lo cual actualmente no es una tecnología muy utilizada en la realización de time-course o cinéticas prolongadas.

Los resultados obtenidos fueron relacionados y organizados de una forma jerárquica, utilizando la ontología, el cual es un término filosófico en donde se estudia la manera en que se relacionan las entidades existentes (Hofweber 2009). La ontología en biología, representa un medio formal en el que se vinculan las relaciones anotadas o no, entre un grupo de conceptos y sus propiedades determinando dominios. En el ámbito de la genómica, el proyecto de Ontología génica “Gene Ontology-GO” es uno de los vocabularios controlados y/o anotados más utilizados para describir la función génica. En este trabajo, hemos utilizado este término con el fin de elucidar la funcionalidad de la expresión transcriptómica; además de la enciclopedia de genes y genomas de Kyoto (KEGG), la cual proporciona una anotación ontológica biológica.

El proyecto GO adscribe un gen a una de estas tres categorías: función molecular, compartimento celular o proceso biológico. Así, la definición inicial del término GO para cada uno de los genes seleccionados son construidos jerárquicamente a partir de la asignación de códigos descriptivos relativos a las categorías mencionadas, de manera que cada término o clase del conjunto puede asociarse a diversas subclases en una jerarquía de inclusión que puede definirse técnicamente como un grafo directo acíclico. Mientras, la búsqueda y caracterización de cada gen mediante la base de datos KEGG proporciona una funcionalidad dentro de un pathway, para ello, KEGG proporciona una herramienta llamada KAAS (Moriya et al. 2007), la cual permite realizar varios tipos de Blast (Basic Local Alignment Search Tool) con el fin de encontrar las regiones de similitud entre las secuencias (Altschul et al. 1990). En nuestro caso, hemos seleccionado la herramienta de Blast llamada Blastx (Boratyn et al. 2013; Johnson et al. 2008), que permite identificar proteínas codificadas por una secuencia de nucleótidos (traducción) utilizando las bases de datos del NCBI. Aquí, las secuencias proporcionadas fueron los genes de trucha arco iris, con el fin de identificar la proteína codificada y su gen en otra especie, en otras palabras, se utilizó Blastx con el fin de obtener el gen ortólogo (genes semejantes por pertenecer a especies con un antepasado común) en una especie soportada en la base de datos de KEGG, como lo es el pez cebra o el humano; con el único objetivo de localizar nuestros genes de interés (genes de trucha arco iris estadísticamente diferentes entre las distintas condiciones) dentro de pathways previamente definidos.

Para este trabajo se empleó una técnica bastante novedosa en este campo, por lo cual el primer paso fue determinar los parámetros a utilizar para el análisis de los datos obtenidos a través de secuenciación masiva (RNA-seq), una vez establecido que la mejor opción en nuestro caso correspondía al uso del transcriptoma dividido en dos secciones (Ohnologos y No ohnologos) como referencia de alineación para nuestros datos de RNA-seq (lecturas o reads), y que la herramienta Bowtie2 fue la optima, se precedió al análisis de los datos comparando los peces infectados respecto a los peces no infectados o controles (peces sanos) tanto al día 3 como 7.

El utilizar como referencia de alineación el transcriptoma dividido en genes ohnologos (son genes paralogos, originados por un proceso de duplicación del genoma completo GTD) y no-ohnologos (genes sin ninguna duplicación o genes únicos) (Brieuc et al. 2014), también fue motivado debido a que un problema adicional de las aproximadamente 23.600 especies de teleósteos actuales (mitad de las especies de vertebrados) es la poliploidia originada hace aproximadamente 230 millones de años (MYA), como consecuencia de al menos dos episodios de duplicación genómica y diversos eventos independientes de duplicación (Volff 2005). Este fenómeno explica la diversidad funcional-estructural en la expresión y tasa de sustitución genéticas que responden, con la abundancia de elementos móviles transponibles en los teleósteos (Krasnov et al. 2005), lo cual les concede una característica especifica de “plasticidad” inmunológica que requiere un esfuerzo considerable en la metodología del procesamiento de datos de expresión génica.

Luego del análisis de los datos de RNA-seq, obtuvimos un total de 6875 transcritos estadísticamente diferenciados (DETs) en los peces infectados luego de 3 días de infección respecto a los peces control y 5875 DETs en los peces infectados luego de 7 días de infección respecto al control. 3141 DETs de estos transcritos fueron comunes tanto a los 3 como a los 7 días post infección. A los 3 días post-infección, se observo más cantidad de genes estimulados respecto a los 7 días, por otra parte, respecto a los DETs con un fold-change >2 y <0.05 encontrados únicamente a a los 3 dias post infección respecto al control, se encontraron 198 DETs de los cuales, mucho de ellos están relacionados con genes correspondientes a la respuesta inmune innata como por ejemplo el gen IFNa. Respecto a los DETs encontrados únicamente al día 7 post-infección, la mayoría de sus interacciones huésped-virus siguen sin investigarse en peces, sin embargo si se han caracterizado algunas de sus funciones en mamíferos, siendo algunos de ellos relacionados con procesos de cáncer, inflamación y daño tisular.

En relación a la anotación funcional de acuerdo con el análisis KO, se observaron 340 DETs con cambios de expresión de ≥2 o ≤ 0,5 veces respecto el control tanto a los 3 como a los 7 dpi., de ellos se encontraron 58 vías o pathways (KO) con diferentes niveles de expresión, siendo uno de los mas enriquecidos los relacionados con metabolismo de aminoácidos, inflamación, proceso celular e inmunológico. A demás, 32 DETs con niveles de expresión similares en las dos fechas muestreadas (3 y 7 dpi.) fueron también involucrados en pathways metabólicos. Respecto a las vías obtenidas exclusivamente a los 3 dpi, se observaron 170 pathways, los cuales tuvieron niveles de regulación más variantes respecto a los 30 pathways observados exclusivamente a los 7 dpi; por otra parte, las vías observadas a los 3 dpi fueron relacionadas con la defensa del huésped e inducción de etapas antivirales.

Como resumen se puede mencionar que a los 3 dpi, gran parte de los DETs que se encontraron estimulados, estaban relacionados con inmunidad innata como lo fueron los IFN-a e ILs mientras que a los 7 dpi, los DETs más representativos fueron involucrados en daño tisular, inflamación, tráfico intracelular de la membrana y alteraciones en el intercambio de los iones de Na^{+}/Ca^{+}. A demás, se identificaron tanto los DETs que se encuentran diferencialmente modulados en su expresión a los 3 y 7 dias, como los DETs que son estimulados con la infección, sin embargo su expresión no es modulada a los 3 y días post-infección. También se identificaron los DETs involucrados exclusivamente a los 3 o 7 días post-infección. Finalmente, se enfatizo en algunas rutas (pathways) inmunológicos como TLR, JAK-STAT, apoptosis entre otros. Sin embargo, para comprender en profundidad los factores que afectan a la expresión de este tipo de grupos de genes requiere más trabajo.

Por último, gracias a la gran explosión de métodos y tecnologías en la experimentación genómica y transcripcional, el estudio de los patrones de expresión génica obtenidos a través de técnicas de secuenciación masiva, microarray o incluso qPCR, se pueden realizar estudios que permiten explorar la compleja conectividad funcional entre los genes sometidos a condiciones experimentales clásicas (control vs. tratamiento) (Shaffer et al. 2006). Sin embargo, debido a la complejidad y, especialmente, a la diversidad genómica incluso dentro de los individuos de la misma especie, a demás, que la mayoría de los genes presentan un solapamiento funcional considerable originando una alta plasticidad fenotípica, es decir, la propiedad que presenta un genotipo de producir más de un fenotipo cuando el organismo se halla en diferentes condiciones ambientales (Pigliucci, Murren, and Schlichting 2006; Whitman and Agrawal 2009), los perfiles de expresión génica inmunitaria pueden servir como guía acerca de la respuesta propia del hospedador, sin embargo no pueden tomarse como niveles (absolutos) plenamente cuantitativos y rígidos.

Una vez se han definido los transcritos o genes implicados en algún proceso de interés, se utilizan varias herramientas como Blast con el fin de agrupar estos datos en categorías funcionales que permitan el análisis del papel biológico de genes del hospedador frente a varios estímulos (vacunación y/o infección), contribuyendo al conocimiento de las características funcionales de los genes involucrados en la respuesta fisiológica y patológica, delimitando un tiempo inmunitario. Por lo cual, estos estudios aquí reportados en esta tesis doctoral ayudan a enriquecer la información en especies que, como en el caso de los salmónidos, carecen, por el momento, de una caracterización génica completamente anotada y su regulación.

Conclusiones

La vacuna DNA que codifica el gen VP2 del virus de la Necrosis Pancreática Infecciosa encapsulada en alginato y administrada por vía oral:

1. No se inactiva en el tracto gastrointestinal. El transcrito VP2 se expresa en riñón anterior bazo, intestino y branquias.

2. Induce la expresión de genes de la respuesta inmune en trucha arco iris y en riñón anterior hay mayor respuesta transcripcional que en ciego pilórico.

3. Induce alta protección que es precedida por una compleja y específica respuesta inmune en órganos internos y locales.

4. Imita cualitativamente los perfiles transcriptómicos inducidos por el virus aunque a menores niveles excepto en interferón 1 que es similar.

5. Modula la transcripción de numerosas quimioquinas, de MHCII y TNFα, moviliza células IgM^+, IgT^+ y linfocitos T $CD3^+$ en diferentes segmentos del tracto intestinal.

6. Estimula células B en el tejido adiposo de trucha, asociado al ciego pilórico.

7. Incorporada al pienso proporciona un método de administración eficaz y práctico para proteger frente a la Necrosis Pancreática Infecciosa en trucha.

8. Ofrece mejores resultados de protección que la vacuna pcDNA encapsulada en quitosano-alginato y administrada por vía oral.

9. Previene en trucha el estado de persistencia y la formación de portadores del virus de la necrosis pancreática infecciosa.

10. La vacuna DNA que codifica el gen G del virus de la necrosis hematopoyética infecciosa en un plásmido de expresión “todo pez” (pIRF1A-G), cuando es encapsulada en alginato y administrada oralmente a truchas, induce respuesta inmune dosis-dependiente y, a dosis altas, protección significativa frente a virus IHNV de alta virulencia.

11. Los perfiles de transcripción en trucha arco iris infectadas con el virus de la necrosis hematopoyética infecciosa y analizados en estado de infección asintomática y clínica, muestran una respuesta inmune muy coordinada en riñón, con mayor número de transcritos diferencialmente expresados a los 3 días post-infección y con rutas de expresión más enriquecidas en genes.

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